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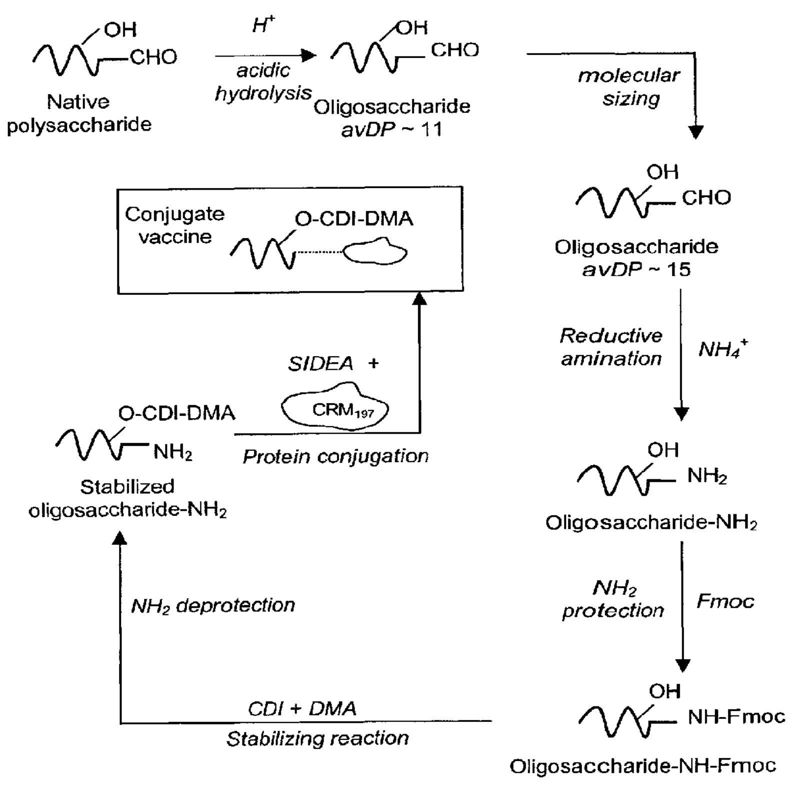

(57) Abstract: A modified polysaccharide, in particular a modified Neisseria meningitides serogroup A polysaccharide, which retains immunogenicity but has improved stability. Typically, the modified polysaccharide is prepared by reacting a capsular polysaccharide, or oligosaccharide fragment thereof, with a bifunctional reagent such as CDI, followed by reaction with an amino compound, such dimethylamine. Modified polysaccharide-protein conjugates and vaccines prepared from such conjugates are also described.

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MODIFIED SACCHARIDES HAVING IMPROVED STABILITY IN WATER

. All documents cited herein are incorporated by reference in their entirety.

TECHNICAL FIELD

This invention is in the field of polysaccharide chemistry and relates to modified saccharides, processes for their preparation, and conjugated derivatives. In particular, the invention relates to modified saccharides having improved stability in water.

BACKGROUND ART

Polysaccharides are important biological molecules and they have been widely used in the pharmaceutical industry for the prevention and treatment of diseases. For example, capsular polysaccharides have been used for many years in vaccines against capsulated bacteria, such as meningococcus (*Neisseria meningitidis*), pneumococcus (*Streptococcus pneumoniae*) and Hib (*Haemophilus influenzae* type B).

To enhance immunogenicity of these polysaccharides, particularly in children, conjugate vaccines were developed. These comprise a capsular polysaccharide conjugated to a carrier protein [*e.g.* references 1, 2, 3]. Conjugation can make T-independent antigens into T-dependent antigens.

A problem with many types of polysaccharide is poor stability in water. The stability of polysaccharides in water depends on the nature of the O-glycosidic bonds joining the saccharide units. Poor stability in water is a result of the O-glycosidic bonds being readily hydrolysed in the presence of acids or glycosidases. The capsular polysaccharide of serogroup A meningococcus is an example of a polysaccharide having poor stability in water.

The stability of polysaccharides is a particular problem in the manufacture of conjugate vaccines. In order to prepare a polysaccharide-protein conjugate, it is necessary to manipulate chemically functional groups on the polysaccharide so that the polysaccharide may be linked to a protein. The polysaccharide may be linked either directly to the protein [2, 4] or it may be linked via a linker group. Many different types of linker groups have been proposed for linking polysaccharides to proteins [*e.g.* 3, 5].

The exposure of a polysaccharide to chemical reagents, particularly acids, may result in undesirable cleavage of glycosidic linkages and consequent fragmentation of the polysaccharide. Such fragmentation is highly undesirable, causing loss in yields in the synthesis of polysaccharide-protein conjugates.

Polysaccharides which are unstable in this way generally require careful choice of reagents and conditions to circumvent the problems described above. However, this limits the reagents available for manipulating the polysaccharide, thus limiting the range of linkages which may be made between

the polysaccharide and carrier protein. In addition, the instability of these polysaccharides means it is difficult to develop robust procedures, which can be used to prepare vaccines on an industrial scale.

It is an object of the invention to provide ways of modifying capsular saccharides so that they do not suffer from instability problems and maintain immunogenicity.

DISCLOSURE OF THE INVENTION

The invention is based on the discovery that modification of hydroxyl groups on monosaccharide units of capsular saccharides offers improved stability. Modified saccharides obtained by the process of the invention are more stable to hydrolysis than their native saccharide counterparts.

Modified saccharides of the invention

The invention provides a modified capsular saccharide comprising a blocking group at a hydroxyl group position on at least one of the monosaccharide units of the corresponding native capsular saccharide.

The term "modified capsular saccharide" means a saccharide which is obtainable from a native capsular saccharide by suitable modification. Hence, the basic sequence of repeating monosaccharide units in the native capsular saccharide is retained in the modified capsular saccharides of the present invention.

The term "saccharide" encompasses both oligosaccharides (*e.g.* containing from 2 to 39 monosaccharide units) and polysaccharides (*e.g.* containing 40 or more monosaccharide units). As found naturally in bacteria, native capsular saccharides generally take the form of polysaccharides. Polysaccharides may be manipulated to give shorter oligosaccharides. Oligosaccharides may be obtained by purification and/or sizing of the native polysaccharide (*e.g.* by hydrolysis in mild acid, by heating, by sizing chromatography *etc.*).

Typically, the modified saccharides of the present invention are oligosaccharides. Oligo-saccharides may be obtained from polysaccharides by any of the sizing methods described above.

The modified capsular saccharides of this invention are obtainable from native capsular saccharides. However, the present invention is not limited to modified saccharides obtained from native capsular saccharides. The modified capsular saccharides of the present invention may be obtained by other methods, such as total or partial synthesis.

The number of monosaccharide units having blocking groups may vary in the present invention. For example, all or substantially all the monosaccharide units of the corresponding capsular saccharide may have blocking groups. Alternatively, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the monosaccharide units of the corresponding capsular saccharide may have blocking groups. At least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25,

26, 27, 28, 29 or 30 monosaccharide units of the corresponding capsular saccharide may have blocking groups.

Likewise, the number of blocking groups on a monosaccharide unit may vary. For example, the number of blocking groups on a monosaccharide unit may be 1, 2, 3, 4, 5 or 6, preferably 1-4, more preferably 1-2.

In one embodiment, the at least one monosaccharide unit having a blocking group is a non-terminal monosaccharide unit. The term "non-terminal monosaccharide unit" means a monosaccharide unit which is not one of the terminal monosaccharide units in the oligosaccharide/polysaccharide chain.

This invention encompasses modified capsular saccharides wherein all the hydroxyl group positions of the terminal and non-terminal monosaccharide units have a blocking group. However, it is preferred that there is at least one free hydroxyl group or amino group in the modified capsular saccharide of the present invention. A free hydroxyl group or amino group is advantageous because it provides a handle for further reactions of the modified capsular saccharide *e.g.* for conjugation to a carrier molecule. When the modified saccharide contains a free hydroxyl group, it is preferably an anomeric hydroxyl group, preferably a terminal anomeric hydroxyl group. When the modified saccharide contains an amino group, it is preferably derived from an anomeric hydroxyl group. Amino groups are readily accessible from anomeric hydroxyl groups by reductive amination (using, for example, $NaBH_3CN/NH_4Cl$).

The term "amino group" includes groups of the formula $-NH_2$ or $-NH$-E, where E is a nitrogen protecting group. Examples of typical nitrogen protecting groups are described below.

The term "blocking group" means any group which blocks the reactivity of a hydroxyl group. The skilled person will be aware of many different types of blocking group. Preferred blocking groups for hydroxyl groups are groups which are directly accessible via a derivatizing reaction of the hydroxyl group *i.e.* by replacing the hydrogen atom of the hydroxyl group with another group. Suitable derivatives of hydroxyl groups which act as blocking groups are, for example, carbamates, sulfonates, carbonates, esters, ethers (*e.g.* silyl ethers or alkyl ethers) and acetals. Some specific examples of such blocking groups are allyl, Aloc, benzyl, BOM, t-butyl, trityl, TBS, TBDPS, TES, TMS, TIPS, PMB, MEM, MOM, MTM, and THP.

However, the blocking group need not be directly accessible via a derivatizing reaction of the hydroxyl group. The blocking group may completely replace the hydroxyl group. For example, the blocking group may be C_{1-12} alkyl, C_{3-12} alkyl, C_{5-12} aryl, C_{5-12} aryl-C_{1-6} alkyl, NR^1R^2 (where R^1 and R^2 are as defined below), H, F, Cl, Br, CO_2H, $CO_2(C_{1-6}$ alkyl), CN, CF_3, CCl_3 *etc.*

Preferably, the blocking group is an electron-withdrawing group. Without wishing to be bound by theory, it is believed that glycosidic bonds are unstable to hydrolysis due to assistance from an intramolecular nucleophilic attack of a saccharide hydroxyl group on the glycosidic linkage (*i.e.* by

formation of a cyclic intermediate). The greater the nucleophilicity of the hydroxyl group, the greater the tendency for intramolecular nucleophilic attack. An electron-withdrawing blocking group has the effect of delocalizing the oxygen lone pair, thereby decreasing the oxygen nucleophilicity and decreasing the tendency for intramolecular nucleophilic attack.

Preferably, the blocking group is of the formula:

$$\text{-O-X-Y} \qquad \text{or} \qquad \text{-OR}^3$$

wherein

X is C(O), S(O) or SO_2;

Y is C_{1-12} alkyl, C_{1-12} alkoxy, C_{3-12} cycloalkyl, C_{5-12} aryl or C_{5-12} aryl-C_{1-6} alkyl, each of which may optionally be substituted with 1, 2 or 3 groups independently selected from F, Cl, Br, CO_2H, $CO_2(C_{1-6}$ alkyl), CN, CF_3 or CCl_3; or Y is NR^1R^2;

R^1 and R^2 are independently selected from H, C_{1-12} alkyl, C_{3-12} cycloalkyl, C_{5-12} aryl, C_{5-12} aryl-C_{1-6} alkyl; or R^1 and R^2 may be joined to form a C_{3-12} saturated heterocyclic group;

R^3 is C_{1-12} alkyl or C_{3-12} cycloalkyl, each of which may optionally be substituted with 1, 2 or 3 groups independently selected from F, Cl, Br, $CO_2(C_{1-6}$ alkyl), CN, CF_3 or CCl_3; or R^3 is C_{5-12} aryl or C_{5-12} aryl-C_{1-6} alkyl, each of which may optionally be substituted with 1, 2, 3, 4 or 5 groups selected from F, Cl, Br, CO_2H, $CO_2(C_{1-6}$ alkyl), CN, CF_3 or CCl_3;

Preferably, when R^3 is C_{1-12} alkyl or C_{3-12} cycloalkyl, it is substituted with 1, 2 or 3 groups as defined above.

The blocking groups of formula -O-X-Y or -OR^3 may be prepared from hydroxyl groups by standard derivatizing procedures, such as reaction of the hydroxyl group with an acyl halide, alkyl halide, sulfonyl halide *etc.* Hence, the oxygen atom in –O-X-Y is preferably the oxygen atom of the hydroxyl group, while the –X-Y group in –O-X-Y preferably replaces the hydrogen atom of the hydroxyl group.

Alternatively, the blocking groups may be accessible via a substitution reaction, such as a Mitsonobu-type substitution. These and other methods of preparing blocking groups from hydroxyl groups are well known.

More preferably, the blocking group is -OC(O)CF_3 [6] or -OC(O)NR^1R^2.

More preferably, the blocking group is a carbamate group of the formula -OC(O)NR^1R^2, wherein R^1 and R^2 are independently selected from C_{1-6} alkyl. More preferably, R^1 and R^2 are both methyl *i.e.* the blocking group is -OC(O)NMe_2.

Carbamate blocking groups have a stabilizing effect on the glycosidic bond and may be prepared under mild conditions. An example of a process for manipulating a saccharide to provide a carbamate blocking group is described below. However, the invention is not limited to modified saccharides

prepared by the processes exemplified herein, and other processes for preparing modified saccharides of the invention will be readily apparent to the skilled person.

The term "alkyl" is used herein to refer to alkyl groups in both straight and branched forms, The alkyl group may be interrupted with 1, 2 or 3 heteroatoms selected from -O-, -NH- or -S-. The alkyl group may also be interrupted with 1, 2 or 3 double and/or triple bonds. However, the term "alkyl" usually refers to alkyl groups having no heteroatom interruptions or double or triple bond interruptions. Where reference is made to C_{1-12} alkyl, it is meant the alkyl group may contain any number of carbon atoms between 1 and 12 (*e.g.* C_1, C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}). Similarly, where reference is made to C_{1-6} alkyl, it is meant the alkyl group may contain any number of carbon atoms between 1 and 6 (*e.g.* C_1, C_2, C_3, C_4, C_5, C_6).

The term "cycloalkyl" includes cycloalkyl, polycycloalkyl, and cycloalkenyl groups, as well as combinations of these with alkyl groups, such as cycloalkylalkyl groups. The cycloalkyl group may be interrupted with 1, 2 or 3 heteroatoms selected from -O-, -NH- or -S-. However, the term "cycloalkyl" usually refers to cycloalkyl groups having no heteroatom interruptions Examples of cycloalkyl groups include cyclopentyl, cyclohexyl, cyclohexenyl, cyclohexylmethyl and adamantyl groups. Where reference is made to C_{3-12} cycloalkyl, it is meant that the cycloalkyl group may contain any number of carbon atoms between 3 and 12 (*e.g.* C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}).

The term "aryl" is used herein to refer to an aromatic group, such as phenyl or naphthyl. Where reference is made to C_{5-12} aryl, it is meant that the aryl group may contain any number of carbon atoms between 5 and 12 (*e.g.* C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}).

The term "C_{5-12} aryl-C_{1-6} alkyl" refers to groups such as benzyl, phenylethyl and naphthylmethyl.

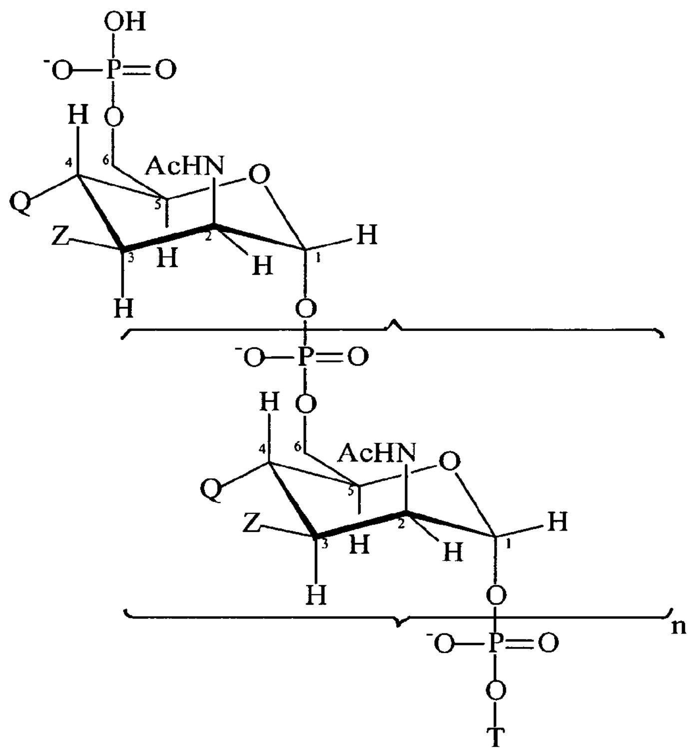
When R^1 and R^2 are joined to form a C_{3-12} saturated heterocyclic group, it is meant that R^1 and R^2 together with the nitrogen atom form a saturated heterocyclic group containing any number of carbon atoms between 3 and 12 (*e.g.* C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}). The heterocyclic group may contain 1 or 2 heteroatoms (such as N, O or S) other than the nitrogen atom. Examples of C_{3-12} saturated heterocyclic groups are pyrrolidinyl, piperidinyl, morpholinyl, piperazinyl, imidazolidinyl, azetidinyl and aziridinyl.

In all the embodiments described above, the modified capsular saccharide is preferably a modified capsular saccharide having phosphodiester linkages. More preferably, the modified capsular saccharide is a modified *Neisseria meningitidis* serogroup A saccharide. *Neisseria meningitidis* serogroup A saccharides are particularly unstable to hydrolysis.

When the modified capsular saccharide is a modified *Neisseria meningitidis* serogroup A saccharide, the blocking group is preferably at the 4 and/or 3-positions, more preferably the 4-position, of the corresponding *Neisseria meningitidis* serogroup A saccharide. Blocking groups in the 4 and/or 3-

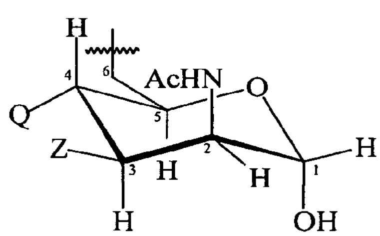
positions *Neisseria meningitidis* serogroup A saccharide have been shown to be particularly efficacious for improving stability towards hydrolysis.

This invention also provides a saccharide of the formula:

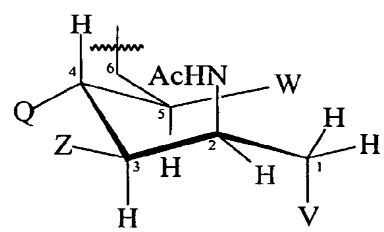


wherein

T is of the formula (A) or (B):



(A)



(B)

n is an integer from 1 to 100;

each Z group is independently selected from –OH or a blocking group as defined above; and

each Q group is independently selected from –OH or a blocking group as defined above;

W is selected from –OH or a blocking group as defined above;

V is selected from $-NH_2$, –NHE, $-NE^1E^2$, –OH, or –O–D, where: E, E^1 and E^2 are nitrogen protecting groups, which may be the same or different, and D is an oxygen protecting group.

and wherein more than about 7% (*e.g.* 8%, 9%, 10% or more) of the Q groups are blocking groups.

Preferably, n is an integer from 15 to 25.

Each of the n+2 Z groups may be the same or different from each other. Likewise, each of the n+2 Q groups may be the same or different from each other.

V is preferably $-NH_2$ or $-NHE$.

Suitable nitrogen protecting groups are silyl groups (such as TMS, TES, TBS, TIPS), acyl derivatives (such as trifluoroacetamides, methoxycarbonyl, ethoxycarbonyl, t-butoxycarbonyl (Boc), benzyloxycarbonyl (Z or Cbz), 9-fluorenylmethoxycarbonyl (Fmoc), 2-(trimethylsilyl)ethoxy carbonyl, allyloxycarbonyl (Alloc), 2,2,2-trichloroethoxycarbonyl (Troc)), sulfonyl derivatives (such as â-trimethylsilylethanesulfonyl (SES)), sulfenyl derivatives, C_{1-12} alkyl, benzyl, benzhydryl, trityl, allyl, 9-phenylfluorenyl, *etc.* A preferred nitrogen protecting group is Fmoc.

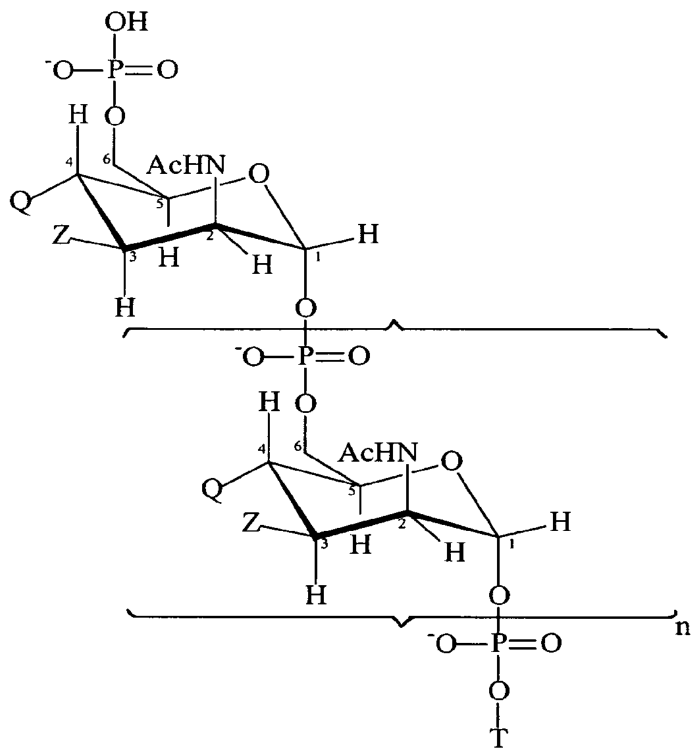
Divalent nitrogen protecting groups, which can be used as E^1E^2, include cyclic imide derivatives (such as N-phthalimides, N-dithiasuccinimides, N-2,3-diphenylmaleimides), imine derivatives (such as N-1,1-dimethylthiomethyleneamines, N-benzylideneamines, N-p-methoxybenzylideneamines, N-diphenylmethyleneamines), enamine derivatives (such as N-(5,5-dimethyl-3-oxo-1-cyclohexenyl)amines), *etc.* A preferred divalent nitrogen protecting group is N-phthalimidyl.

Suitable oxygen protecting groups include esters, ethers (*e.g.* silyl ethers or alkyl ethers) and acetals. Specific examples include allyl, acetyl, Aloc, benzyl, benzyloxymethyl (BOM), t-butyl, trityl, tert-butyldimethylsilyl (TBS), tert-butyldiphenylsilyl (TBDPS), triethylsilyl (TES), trimethylsilyl (TMS), tri-isopropylsilyl (TIPS), paramethoxybenzyl (PMB), MEM, methoxymethyl (MOM), MTM and tetrahydropyranyl (THP).

All the Z groups may be OH. Alternatively, at least 10%, 20, 30%, 40%, 50% or 60% of the Z groups may be OAc. Preferably, about 70% of the Z groups are OAc, with the remainder of the Z groups being OH or blocking groups as defined above.

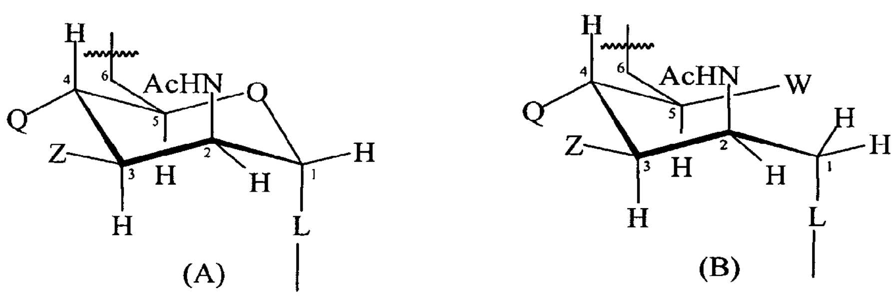
At least about 7% of Q groups are blocking groups. Preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% of the Q groups are blocking groups. Alternatively, all the Q groups may be blocking groups.

The invention also provides a molecule comprising a saccharide moiety of formula:



wherein

T is of the formula (A) or (B):



(A) (B)

n, Z, Q and W are as defined above, and: L is O, NH, NE, S or Se.

The free covalent bond of L can be joined to any appropriate moiety *e.g.* to –H, –E, a linker, a protein carrier, *etc.* L is preferably N or O. It is also possible for L to be N, joined to a divalent linker, to a divalent protecting group, or to a divalent protein carrier.

Process for producing a modified saccharide

The invention provides a process for modifying a capsular saccharide comprising the steps of:

(a) providing a capsular saccharide having at least one hydroxyl group on a monosaccharide unit; and

(b) converting said at least one hydroxyl group into a blocking group.

The blocking group may be any of the blocking groups defined above.

The capsular saccharide may be a native capsular saccharide (oligosaccharide or polysaccharide). As an alternative, the capsular saccharide may be, for example, a de-O-acetylated capsular saccharide and/or a capsular saccharide having a terminal amino group (*e.g.* obtained by reductive amination).

A preferred process for modifying a saccharide wherein the blocking group is $-OC(O)NR^1R^2$ is when step (b) comprises the steps of:

(b1) reacting the capsular saccharide with a bifunctional reagent in an organic solvent; and

(b2) reacting the product of step (b1) with an amino compound of formula (I):

$$HNR^1R^2 \qquad (I)$$

wherein R^1 and R^2 are as defined above.

The term "bifunctional reagent" means any reagent which is capable of performing the dual functions of (i) providing in step (b1) a first electrophilic carbon atom for coupling with the hydroxyl group(s) on the saccharide; and (ii) providing a second electrophilic carbon atom for coupling with the amino group used in step (b2). Generally, the second electrophilic carbon atom is regenerated from the first electrophilic carbon atom during step (b). The bifunctional reagent provides a -C(O)- linkage between the polysaccharide and the amino compound.

Bifunctional reagents for use in the present invention include, but are not limited to, 1,1'-carbonyldiimidazole (CDI), carbonyl di-1,2,4-triazole (CDT), carbonyl di-1,2,3-benzotriazole (CDB), diphenylcarbonate, cyanogen bromide, phosgene or triphosgene. The skilled person will be aware of other bifunctional reagents which can perform the same function as these.

A preferred bifunctional reagent is CDI. CDI has the advantage of being a milder reagent than, for example, phosgene or cyanogen bromide. In particular, coupling reactions using CDI do not generate hydrohalic acid gases, such as HCl or HBr. The generation of HCl or HBr gas is undesirable, because these gases require scrubbing of the reaction chamber outlet to avoid their escape into the atmosphere. Moreover, the generation of HCl or HBr gas may affect sensitive functional groups on the saccharide, resulting in loss in yields due to decomposition or fragmentation of the saccharide.

The organic solvent used in step (b1) is preferably an aprotic solvent. Aprotic solvents are well known to the person skilled in the art and do not contain any ionizable hydrogen atoms. These solvents are advantageous because they facilitate the reaction of hydroxyl group(s) on the saccharide with the bifunctional reagent, by enhancing the nucleophilicity of the hydroxyl group(s). Suitable aprotic solvents include, but are not limited to, dimethylsulfoxide (DMSO), dimethylformamide (DMF), formamide, hexamethylphosphorus triamide (HMPT), 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU), dimethylacetamide (DMAC), or hexamethylphosphoramide (HMPA). DMSO is preferred.

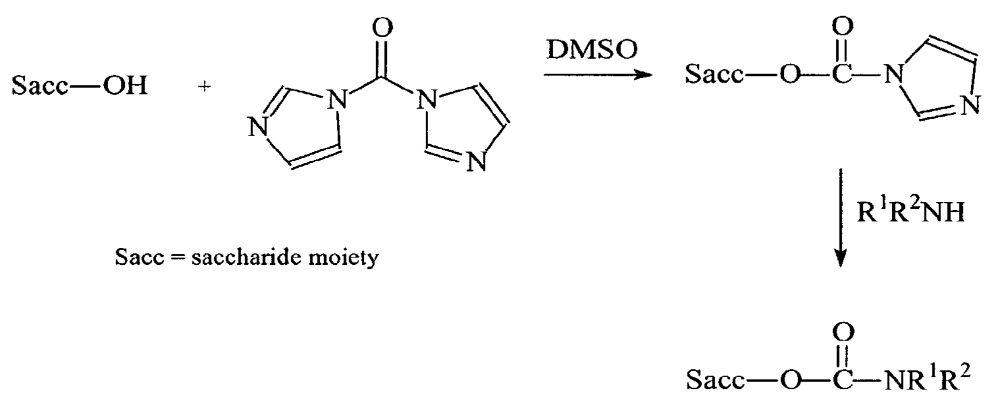
In step (b2) of the process of the invention, the product of step (b1) is reacted with an amino compound to form the modified polysaccharide. The amino compound used in the process of the

present invention is of formula (I), as defined above. In formula (I), preferably, R^1 and R^2 are independently selected from C_{1-6} alkyl. More preferably R^1 and R^2 are both methyl.

Suitable amino compounds which may be used in the invention are methylamine, dimethylamine, ethylamine, *N*-ethylmethylamine, diethylamine, *N*-methylpropylamine, *N*-ethylpropylamine, isopropylamine, butylamine, *N*-methylbutylamine, *N*-ethylbutylamine, *N*-propylbutylamine, *N*-methylcyclopentylamine, *N*-ethylcyclopentylamine, cyclohexylamine, *N*-methylcyclohexylamine, *N*-ethylcyclohexylamine, benzylamine, *N*-ethylbenzylamine, *N*-methylbenzylamine, isobutylamine, tert-butylamine, cyclopentylamine, dibenzylamine, pyrrolidine, piperidine, morpholine, piperazine, imidazolidine, azetidine, aziridine, aniline, *N*-methylaniline and *N*-ethylaniline. These may be used in the salt form (*e.g.* hydrochloride salt).

Preferably, the amino compound used in the present invention is a secondary amine. More preferably, the amine is dimethylamine.

A preferred process of the invention is exemplified in Scheme 1 below:



Scheme 1

In this scheme, the saccharide (*e.g.* MenA polysaccharide or oligosaccharide) is first activated through at least one of its hydroxyl groups on a monosaccharide unit using CDI in DMSO solvent. The resulting imidazole carbamate intermediate is trapped by the amine R^1R^2NH (*e.g.* dimethylamine) to give the modified saccharide.

The modified saccharides may alternatively be prepared in a one-step process by reacting one or more hydroxyl groups on a capsular saccharide with a reagent of the formula $XC(O)NR^1R^2$, wherein X is a leaving group, and R^1 and R^2 are as defined above. Suitable leaving groups include, but are not limited to, -Cl, -Br, $-CF_3$, $-OC_6F_5$ or $-CCl_3$.

Alternatively, modified capsular saccharides of the present invention may be prepared by synthetic means, for example, from suitable monosaccharide units. Typically, total synthesis of a modified capsular saccharide comprises forming glycosidic linkages (*e.g.* phosphodiester linkages) between

suitable monosaccharide units and then modifying the resultant saccharide in any manner described above. Alternatively, the monosaccharide units may be modified before forming the glycosidic linkages to provide the same modified capsular saccharide.

The modified capsular saccharides of this invention are preferably oligosaccharides. Starting from native capsular polysaccharides, modified capsular oligosaccharides may be obtained by either of two methods: (1) modifying the capsular polysaccharide followed by sizing the modified polysaccharide to form a modified oligosaccharide; or (2) sizing the capsular polysaccharide followed by modifying the resultant oligosaccharide to form a modified oligosaccharide. Both methods are encompassed within the present invention. However, the first method is preferred, since this method ensures that a terminal hydroxyl group will be available for subsequent conjugation of the modified oligosaccharide to a carrier molecule, such as a protein.

The present invention also provides a process for modifying a *Neisseria meningitidis* serogroup A polysaccharide comprising the steps of:

(a) providing a *Neisseria meningitidis* serogroup A polysaccharide;
(b) sizing said polysaccharide to provide an oligosaccharide; and
(c) converting at least one hydroxyl group of the oligosaccharide into a blocking group, as described above.

Step (b) of this process may optionally be followed by known derivatizing step(s) before step (c). Known derivatizing steps include, for example, reductive amination followed by protection of the resulting -NH_2 group and/or de-O-acetylation.

This invention also provides a process for modifying a *Neisseria meningitidis* serogroup A polysaccharide comprising the steps of:

(a) providing a *Neisseria meningitidis* serogroup A polysaccharide;
(b) converting at least one hydroxyl group of the polysaccharide into a blocking group, as described above; and
(c) sizing the resulting polysaccharide to provide an oligosaccharide.

Step (c) of this process may optionally be followed by known derivatizing step(s). Known derivatizing steps include, for example, reductive amination followed by protection of the resulting -NH_2 group and/or de-O-acetylation.

Any of the processes described above may be followed by a step in which contaminants (*e.g.* low molecular weight contaminants) are removed.

Capsular saccharide starting materials

The modified capsular saccharides of the invention are obtainable from native capsular saccharides. The term "native capsular saccharide" refers to sugar-containing polymers (*e.g.* polymers of sugars, sugar acids, amino sugars, polyhydric alcohols, sugar alcohols, and sugar phosphates *etc.*) which may

be found in the capsule of bacteria (both Gram-positive and Gram-negative) such as *N.meningitidis*, *S.pneumoniae* and *H.influenzae*. Furthermore, "native capsular saccharide" includes both polysaccharides and oligosaccharides. Native capsular oligosaccharides may be obtained by sizing native polysaccharides.

The "hydroxyl group position" of a native capsular saccharide is a position on the native capsular saccharide having a hydroxyl group. However, it does not include positions in glycosidic linkages, or the residues thereof, having hydroxyl groups (*e.g.* a hydroxyl group which is part of a phosphate linkage does not occupy a hydroxyl group position). Nor does it include positions occupied by an anomeric hydroxyl group on a terminal monosaccharide unit. Positions where there is an acetoxy group (AcO) group on the native capsular saccharide are also not hydroxyl group positions.

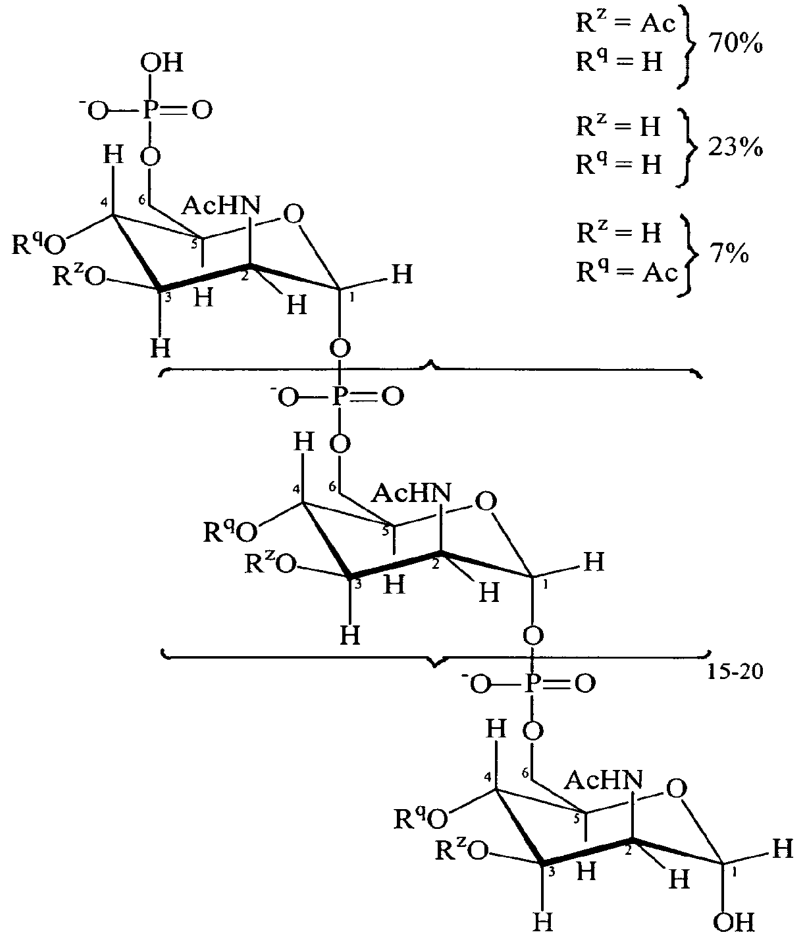
The native capsular saccharide may comprise saccharide units linked by phosphodiester bonds. Saccharides comprising phosphodiester bonds are unstable to hydrolysis.

The native capsular saccharide and the modified capsular saccharide of the invention are preferably immunogenic in mammals (*e.g.* humans). The mammal may be a human adult or a child.

The native capsular saccharide is preferably a polysaccharide (or oligosaccharide fragment thereof) from *N.meningitidis* (including serogroups A, B, C, W135 and Y), *S.pneumoniae* (including serotypes 1, 4, 5, 6B, 9V, 14,18C, 19F and 23F), *H.influenzae* type B, *Neisseria gonorrhoeae, Streptococcus agalactiae, Escherichia coli, Salmonella typhi, Streptococcus mutans, Cryptococcus neoformans, Moraxella catarrhalis, Klebsiella pneumoniae, Staphylococcus aureus,* and/or *Pseudomonas aeruginosa*.

Although the invention may be applied to any serogroup of *N.meningitidis*, it is preferred to use a capsular saccharide from serogroup A ("MenA"). The MenA capsular saccharide is particularly unstable in aqueous solution, meaning that special procedures need to be used to perform chemical manipulations (*e.g.* conjugation to carrier proteins) on this molecule. However, MenA saccharides modified according to the invention are found to be advantageously stable in aqueous solution.

The MenA capsular polysaccharide $\{\rightarrow 6)\text{-D-Man}p\text{NAc}(3/4\text{OAc})\text{-}\alpha\text{-}(1\rightarrow \text{OPO}_3\rightarrow\}$ is composed of N-acetylmannosamine residues linked together by α1-6 phosphodiester bonds having the repeat units shown below.



In accordance with the definitions above, 93% of the 4-positions are hydroxyl group positions, and 30% of the 3-positions are hydroxyl group positions. The terminal 1-hydroxy group also occupies a hydroxyl group position. The terminal 1-hydroxy group is a terminal anomeric hydroxyl group. The hydroxyl group which is part of the -OP(O)(OH)O$^-$ group is not a hydroxyl group position.

Saccharide-protein conjugates

The modified saccharides of the invention may be subjected to any usual downstream processing which is applied to saccharides (*e.g.* derivatisation, conjugation, fragmentation, *etc.*). To enhance immunogenicity, modified saccharides of the invention are preferably conjugated to a carrier protein. Conjugation to carrier proteins is particularly useful for paediatric vaccines [7] and is a well known technique [*e.g.* reviewed in refs. 8 to 16 *etc.*].

The invention thus provides a conjugate of a protein and a modified saccharide of the invention. The protein may be conjugated to the saccharide directly, or a linker may be used. Any suitable linker chemistry can be used. The improved stability of the modified polysaccharide advantageously allows a wide range of linkages to be used.

As described above, it is preferred that the modified capsular saccharide has at least one free hydroxyl group or amino group which can be used as a handle for subsequent linkage to a carrier protein.

A modified capsular saccharide having a free hydroxyl group may be obtained by selectively blocking hydroxyl groups on a capsular saccharide, or selectively de-blocking a modified capsular saccharide in which all the hydroxyl groups are blocked. Alternatively, a free hydroxyl group may be revealed by sizing a modified capsular saccharide. Preferably, the at least one free hydroxyl group is a terminal anomeric hydroxyl group. The terminal anomeric hydroxyl group is preferred as the free hydroxyl group because a terminal anomeric hydroxyl group may be revealed by sizing a modified capsular saccharide.

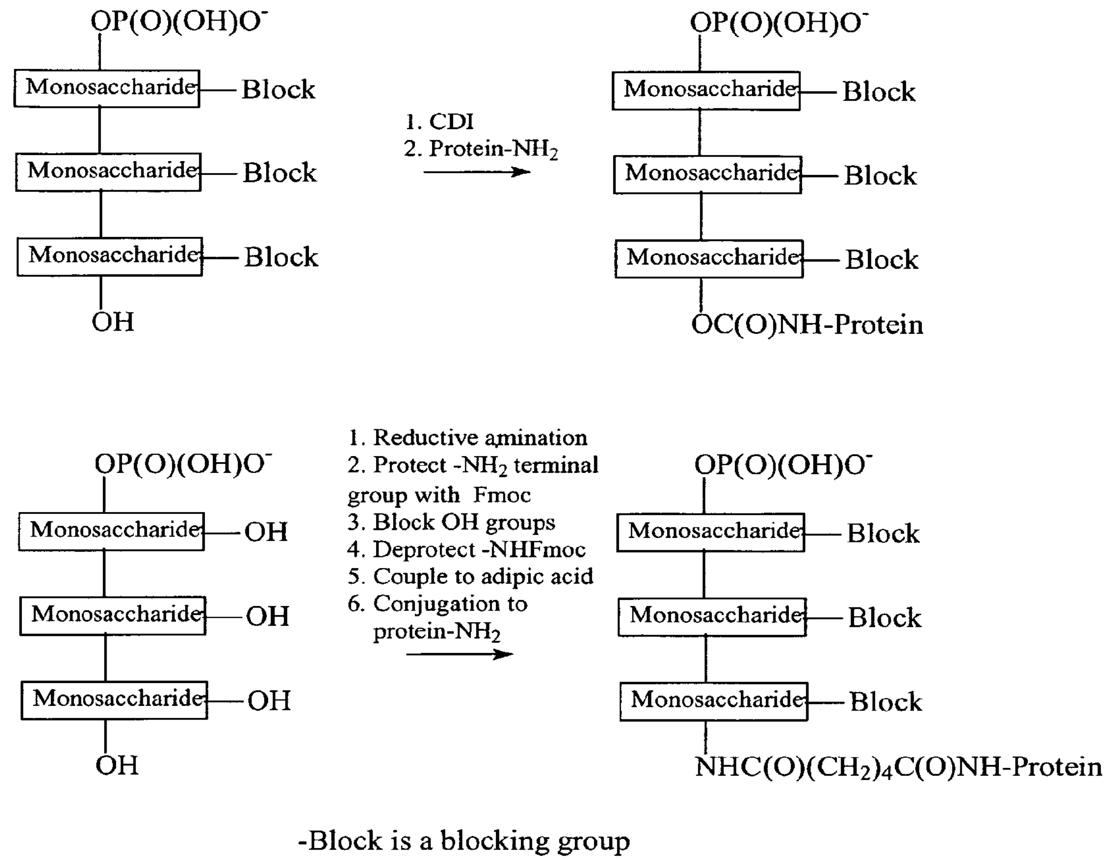
A modified capsular saccharide having a free amino group may be obtained by reductive amination of a terminal anomeric hydroxyl group, optionally followed by protection of the resulting $-NH_2$ group. The reductive amination reaction may be carried out before or after the modifying step of the present invention. Preferably, reductive amination is carried out before the modifying step of the present invention since the resulting $-NH_2$ group can be selectively protected/deprotected in the presence of hydroxyl groups/blocking groups.

Direct linkages to the protein may comprise oxidation of the polysaccharide followed by reductive amination with the protein, as described in, for example, references 2 and 4.

Linkages via a linker group may be made using any known procedure, for example, the procedures described in references 3 and 5. A preferred type of linkage is a carbonyl linker, which may be formed by reaction of a free hydroxyl group of the modified saccharide with CDI [17, 18] followed by reaction with a protein to form a carbamate linkage. Another preferred type of linkage is an adipic acid linker, which may be formed by coupling a free $-NH_2$ group on the modified saccharide with adipic acid (using, for example, diimide activation), and then coupling a protein to the resulting saccharide-adipic acid intermediate. [12, 19, 20]. Other linkers include B-propionamido [21], nitrophenyl-ethylamine [22], haloacyl halides [23], glycosidic linkages [24], 6-aminocaproic acid [25], ADH [26], C_4 to C_{12} moieties [27] *etc.*

Conjugation may involve: reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group; reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate; and coupling the CDI carbamate intermediate with an amino group on a protein.

Scheme 2 shows two different examples of how a capsular saccharide may be conjugated to a carrier protein, in accordance with the present invention. In the first example, the protein is conjugated via a terminal hydroxyl group. In the second example, the protein is linked via a terminal amino group.



Scheme 2

Preferred carrier proteins are bacterial toxins or toxoids, such as diphtheria or tetanus toxoids. These are commonly used in conjugate vaccines. The CRM_{197} diphtheria toxoid is particularly preferred [28]. Other suitable carrier proteins include the *N.meningitidis* outer membrane protein [29], synthetic peptides [30,31], heat shock proteins [32,33], pertussis proteins [34,35], protein D from *H.influenzae* [36], cytokines [37], lymphokines [37], hormones [37], growth factors [37], toxin A or B from *C.difficile* [38], iron-uptake proteins [39] *etc.* It is possible to use mixtures of carrier proteins.

After conjugation, free and conjugated saccharides can be separated. There are many suitable methods, including hydrophobic chromatography, tangential ultrafiltration, diafiltration *etc.* [see also refs. 40, 41 *etc.*].

A single carrier protein may carry multiple different saccharides [42].

Pharmaceutical compositions and methods

The invention provides a pharmaceutical composition comprising (a) a modified saccharide of the invention and/or a conjugate of the invention, and (b) a pharmaceutically acceptable carrier.

Where a conjugate is present, the composition may also comprise free carrier protein [43].

'Pharmaceutically acceptable carriers' include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, trehalose [44] lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. The vaccines may also contain diluents, such as water, saline, glycerol, *etc.* Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences.

Typically, the compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect. Direct delivery of the compositions will generally be parenteral (*e.g.* by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly, or delivered to the interstitial space of a tissue). The compositions can also be administered into a lesion. Other modes of administration include oral and pulmonary administration, rectal (suppositories), and transdermal or transcutaneous applications [*e.g.* ref. 45], needles, and hyposprays. Dosage treatment may be a single dose or a multiple dose schedule (*e.g.* including booster doses).

The composition of the invention is preferably sterile, buffered, and/or pyrogen-free.

The composition is preferably an immunogenic composition (*e.g.* a vaccine). Vaccines based on saccharides or saccharide-protein conjugates are well known in the art.

Immunogenic compositions comprise an immunologically effective amount of saccharide antigen, as well as any other of other specified components, as needed. By 'immunologically effective amount', it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (*e.g.* non-human primate, primate, *etc.*), the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Dosage treatment may be a single dose schedule or a multiple dose schedule (*e.g.* including booster doses). The vaccine may be administered in conjunction with other immunoregulatory agents.

The immunogenic composition may include an adjuvant. Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (A) aluminium compounds *e.g.* aluminium hydroxides (*e.g.* oxyhydroxides), aluminium phosphates (*e.g.* hydroxyphosphates, orthophosphates), aluminium sulphates, *etc.* [*e.g.* see chapters 8 & 9 of ref. 46]), or mixtures of

different aluminium compounds, with the compounds taking any suitable form (*e.g.* gel, crystalline, amorphous *etc.*), and with adsorption being preferred; (B) MF59 (5% Squalene, 0.5% Tween 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer) [see Chapter 10 of ref. 46; see also ref. 47]; (C) liposomes [see Chapters 13 and 14 of ref. 46]; (D) ISCOMs [see Chapter 23 of ref. 46], which may be devoid of additional detergent [48]; (E) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion [see Chapter 12 of ref. 46]; (F) Ribi™ adjuvant system (RAS), (Ribi Immunochem) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™); (G) saponin adjuvants, such as QuilA or QS21 [see Chapter 22 of ref. 46], also known as Stimulon™; (H) chitosan [*e.g.* 49]; (I) complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA); (J) cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, *etc.*), interferons (*e.g.* interferon-ã), macrophage colony stimulating factor, tumor necrosis factor, *etc.* [see Chapters 27 & 28 of ref. 46]; (K) microparticles (*i.e.* a particle of ~100nm to ~150ì m in diameter, more preferably ~200nm to ~30ì m in diameter, and most preferably ~500nm to ~10ì m in diameter) formed from materials that are biodegradable and non-toxic (*e.g.* a poly(á-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone *etc.*); (L) monophosphoryl lipid A (MPL) or 3-O-deacylated MPL (3dMPL) [*e.g.* chapter 21 of ref. 46]; (M) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions [50]; (N) oligonucleotides comprising CpG motifs [51] *i.e.* containing at least one CG dinucleotide, with 5-methylcytosine optionally being used in place of cytosine, and/or CI motif; (O) a polyoxyethylene ether or a polyoxyethylene ester [52]; (P) a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol [53] or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol [54]; (Q) an immunostimulatory oligonucleotide (*e.g.* a CpG oligonucleotide) and a saponin [55]; (R) an immunostimulant and a particle of metal salt [56]; (S) a saponin and an oil-in-water emulsion [57]; (T) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol) [58]; (U) *E.coli* heat-labile enterotoxin ("LT"), or detoxified mutants thereof, such as the K63 or R72 mutants [*e.g.* Chapter 5 of ref. 59]; (V) cholera toxin ("CT"), or detoxified mutants thereof [*e.g.* Chapter 5 of ref. 59]; and (W) monophosphoryl lipid A mimics, such as aminoalkyl glucosaminide phosphate derivatives *e.g.* RC-529 [60]; (X) polyphosphazene (PCPP); (Y) a bioadhesive [61] such as esterified hyaluronic acid microspheres [62] or a mucoadhesive selected from the group consisting of cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrollidone, polysaccharides and carboxymethylcellulose; or (Z) other substances that act as immunostimulating agents to enhance the effectiveness of the composition [*e.g.* see Chapter 7 of ref. 46]. Alum (especially aluminium phosphates and/or hydroxides) is a preferred adjuvant.

Muramyl peptides include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE), *etc.*

Once formulated, the compositions of the invention can be administered directly to the subject. The subjects to be treated can be animals; in particular, human subjects can be treated. The vaccines are particularly useful for vaccinating children and teenagers.

Vaccines according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat disease after infection), but will typically be prophylactic.

As well as modified saccharides, the composition may comprise further antigenic components. For instance, the composition may include one or more further saccharides (whether or not modified according to the invention). For instance, the composition may comprise saccharides from serogroups C, W135 and Y of *N.meningitidis* (*e.g.* in addition to a modified MenA saccharide). These will typically be conjugated to carrier proteins, and saccharides from different serogroups of *N.meningitidis* may be conjugated to the same or different carrier proteins. Where a mixture comprises capsular saccharides from both serogroups A and C, it is preferred that the ratio (w/w) of MenA saccharide:MenC saccharide is greater than 1 (*e.g.* 2:1, 3:1, 4:1, 5:1, 10:1 or higher). Improved immunogenicity of the MenA component has been observed when it is present in excess (mass/dose) to the MenC component. [63]

The composition may also comprise protein antigens.

Antigens which can be included in the composition of the invention include:

- antigens from *Helicobacter pylori* such as CagA [64 to 67], VacA [68, 69], NAP [70, 71, 72], HopX [*e.g.* 73], HopY [*e.g.* 73] and/or urease.
- a protein antigen from *N.meningitidis* serogroup B, such as those in refs. 74 to 80, with protein '287' (see below) and derivatives (*e.g.* 'ÄG287') being particularly preferred.
- an outer-membrane vesicle (OMV) preparation from *N.meningitidis* serogroup B, such as those disclosed in refs. 81, 82, 83, 84 *etc.*
- a saccharide antigen from *N.meningitidis* serogroup C, such as the oligosaccharide disclosed in ref. 85 from serogroup C [see also ref. 86].
- a saccharide antigen from *Streptococcus pneumoniae* [*e.g.* 87, 88, 89].
- an antigen from hepatitis A virus, such as inactivated virus [*e.g.* 90, 91].
- an antigen from hepatitis B virus, such as the surface and/or core antigens [*e.g.* 91, 92].
- an antigen from hepatitis C virus [*e.g.* 93].
- an antigen from *Bordetella pertussis*, such as pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) from *B.pertussis*, optionally also in combination with pertactin and/or agglutinogens 2 and 3 [*e.g.* refs. 94 & 95].

- a diphtheria antigen, such as a diphtheria toxoid [*e.g.* chapter 3 of ref. 96] *e.g.* the CRM_{197} mutant [*e.g.* 97].
- a tetanus antigen, such as a tetanus toxoid [*e.g.* chapter 4 of ref. 96].
- a saccharide antigen from *Haemophilus influenzae* B [*e.g.* 86].
- an antigen from *N.gonorrhoeae* [*e.g.* 74, 75, 76].
- an antigen from *Chlamydia pneumoniae* [*e.g.* 98, 99, 100, 101, 102, 103, 104].
- an antigen from *Chlamydia trachomatis* [*e.g.* 105].
- an antigen from *Porphyromonas gingivalis* [*e.g.* 106].
- polio antigen(s) [*e.g.* 107, 108] such as IPV or OPV.
- rabies antigen(s) [*e.g.* 109] such as lyophilised inactivated virus [*e.g.*110, RabAvert™].
- measles, mumps and/or rubella antigens [*e.g.* chapters 9, 10 & 11 of ref. 96].
- influenza antigen(s) [*e.g.* chapter 19 of ref. 96], such as the haemagglutinin and/or neuraminidase surface proteins.
- an antigen from *Moraxella catarrhalis* [*e.g.* 111].
- an antigen from *Streptococcus agalactiae* (group B streptococcus) [*e.g.* 112, 113].
- a saccharide antigen from *Streptococcus agalactiae* (group B streptococcus).
- an antigen from *Streptococcus pyogenes* (group A streptococcus) [*e.g.* 113, 114, 115].
- an antigen from *Staphylococcus aureus* [*e.g.* 116].
- an antigen from *Bacillus anthracis* [*e.g.* 117, 118, 119].
- an antigen from a virus in the flaviviridae family (genus flavivirus), such as from yellow fever virus, Japanese encephalitis virus, four serotypes of Dengue viruses, tick-borne encephalitis virus, West Nile virus.
- a pestivirus antigen, such as from classical porcine fever virus, bovine viral diarrhoea virus, and/or border disease virus.
- a parvovirus antigen *e.g.* from parvovirus B19.
- a prion protein (*e.g.* the CJD prion protein)
- an amyloid protein, such as a beta peptide [120]
- a cancer antigen, such as those listed in Table 1 of ref. 121 or in tables 3 & 4 of ref. 122.

The composition may comprise one or more of these further antigens.

Toxic protein antigens may be detoxified where necessary (*e.g.* detoxification of pertussis toxin by chemical and/or genetic means [95]).

Where a diphtheria antigen is included in the composition it is preferred also to include tetanus antigen and pertussis antigens. Similarly, where a tetanus antigen is included it is preferred also to include diphtheria and pertussis antigens. Similarly, where a pertussis antigen is included it is preferred also to include diphtheria and tetanus antigens.

Antigens are preferably adsorbed to an aluminium salt.

Antigens in the composition will typically be present at a concentration of at least 1μg/ml each. In general, the concentration of any given antigen will be sufficient to elicit an immune response against that antigen.

As an alternative to using proteins antigens in the composition of the invention, nucleic acid encoding the antigen may be used [*e.g.* refs. 123 to 131]. Protein components of the compositions of the invention may thus be replaced by nucleic acid (preferably DNA *e.g.* in the form of a plasmid) that encodes the protein.

The invention also provides a method for raising an antibody response in a mammal, comprising administering a pharmaceutical composition of the invention to the mammal. The mammal is preferably a human. The human may be an adult or, preferably, a child. The antibody response is preferably protective against infection by *N.meningitidis* serogroup A.

The invention also provides a method for immunising a mammal, comprising administering a pharmaceutical composition of the invention to the mammal.

This invention also provides a modified saccharide of the invention, or a conjugate of the invention, for use as a medicament.

The invention also provides the use of a modified saccharide of the invention, or of a conjugate of the invention, in the manufacture of a medicament for preventing or treating a disease caused by capsulate bacteria. Diseases caused by *Neisseria* include meningitis, septicaemia and gonorrhoea. Diseases caused by *H.influenzae* include otitis media, bronchitis, pneumonia, cellulitis, pericarditis, and meningitis. Diseases caused by pneumococcus include meningitis, sepsis and pneumonia. The prevention and/or treatment of bacterial meningitis is thus preferred.

Definitions

The term "comprising" means "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term "about" in relation to a numerical value x means, for example, $x \pm 10\%$.

It will be appreciated that sugar rings can exist in open and closed form and that, whilst closed forms are shown in structural formulae herein, open forms are also encompassed by the invention.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the *avDP* of Men A samples after incubation at 37, 49 and 57 °C plotted as a function of time (h).

Figure 2 shows the *avDP* of MenA-CDI-DMA samples after incubation at 37, 49, 57 °C plotted as a function of time (h)

Figure 3 shows a stacked plot of ^{31}P NMR 242.9 MHz spectra of MenA-CDI-DMA samples incubated at 57 °C for 0, 24, 48, 72, 96 hours. Some signal labels are indicated.

Figure 4 shows the *avDP* vs. time diagram to compare colorimetric and ^{31}P NMR analytical methods.

Figure 5 shows the *avDP* of native and modified MenA saccharides at 2-8°C over time

Figure 6 shows labelled ^{1}H NMR 600 MHz spectrum of MenA-CDI-DMA at 298 K. Some assignments are indicated.

Figure 7 shows results of a competitive ELISA test performed using MenA, MenA-CDI and MenA-CDI-DMA oligosaccharides as coating agent. The concentration of competitors ranged from 10^0 to 10^{-7} mg/ml.

Figure 8 illustrates the reaction scheme for conjugation of MenA oligosaccharides.

Figure 9 shows the 600 MHz ^{1}H NMR spectrum at 25°C of activated modified MenA. Some signal labels are indicated.

Figures 10A & 10B show hetero-correlate $^{1}H,^{13}C$ NMR spectra at 25°C of activated modified MenA. In both Figure 10A & 10B, the X axis runs approximately from 5.7ppm at the left to 1.8ppm at the right. In Figure 10A, the Y axis runs approximately from 145ppm at the top to 185ppm at the bottom; in Figure 10B it runs approximately from 5ppm at the top to 105ppm at the bottom.

Figure 11 shows superimposed ^{1}H NMR spectra of activated modified MenA DP4 and activated native MenA DP4 (without the chemical modification by CDI and DMA).

Figure 12 shows a 243 MHz ^{31}P NMR spectrum at 25°C of activated modified MenA.

Figures 13 and 14 show the appearance of free saccharide due to hydrolysis of conjugates stored at 37°C over a four week period. Modified saccharides are shown as squares, natural as empty triangles.

Figure 15 shows the appearance of free saccharide due to hydrolysis of conjugates stored at 37°C for four weeks at various pH. The left-hand column of each pair shows native oligosaccharide.

Figure 16 shows the anti-MenA-pS IgG titres induced by (left to right) lot 3, lot 5 and lot 002011 conjugates. Bars show 95% confidence limits.

Figure 17 shows IgG subclasses analysis of pooled sera from immunization with MenA modified and unmodified conjugates (lots 3, 5 & 002011). Values are OD_{405nm} multiplied by 1000.

Figure 18 shows the results of a competitive ELISA using MenA pS as competitor. The Y axis shows OD_{405nm} values multiplied by 1000. The X axis shows the reciprocal serum dilution. Unmodified MenA oligosaccharide is shown in circles; modified saccharides are shown in squares (lot 3) and in triangles (lot 5). Empty symbols show data without competitor polysaccharide; filled symbols show data in the presence of competitor polysaccharide.

MODES FOR CARRYING OUT THE INVENTION

Modification of MenA oligosaccharide

Capsular polysaccharide was purified from MenA and was hydrolysed to give MenA oligosaccharide. The polysaccharide (2 g) was hydrolyzed at 50°C in 50 mM sodium acetate buffer, pH 4.75, at a polysaccharide concentration of 10 mg/mL for about 4 hours [86]. After hydrolysis, the solution was dried by rotary evaporation.

The oligosaccharide was activated using the reaction scheme shown above in Scheme 1. The oligosaccharide was dissolved in DMSO to give a saccharide concentration of 10 mg/mL. According to a molar ratio of oligosaccharide:CDI being 1:20, 21.262 g of CDI (Sigma™) was then added and the reaction mixture stirred for 16 hours at room temperature. The resulting MenA-CDI compound was purified by selective precipitation in a 80:20 (v/v) acetone:DMSO mixture followed by centrifugation. The efficiency of the activation reaction was calculated to be about 67.9% by determining the ratio of free imidazole to bonded imidazole.

In the second reaction step, the MenA-CDI oligosaccharide was solubilised in DMSO at a saccharide concentration of about 10 mg/mL. According to a molar ratio of MenA-CDI unit:DMA being 1:100, 36.288 g of 99% dimethylamine hydrochloride (Sigma™) was added and the reaction mixture stirred for 16 hours at room temperature. The reaction product was freeze-dried and re-solubilised in 10 mg/mL water solution.

To remove the low molecular weight reaction reagent (in particular the dimethylamine (DMA)) from the oligosaccharide preparation, a dialysis step was performed through a 3.5 kDa MWCO membrane (Spectra/Por®). Four dialysis steps were carried out: (i) 16 hours vs. 2 L of 1 M sodium chloride (dialysis factor 1:20), (ii) 16 hours vs. 2 L of 0.5 M sodium chloride (dialysis factor 1:20), (iii) and (iv) 16 hours vs. 2 L of WFI (dialysis factor 1:20). To improve the purification a diafiltration step was also performed through a 1 kDa MWCO membrane (Centricon™).

The purified MenA-CDI-DMA product was buffered at pH 6.5 in 25 mM L-histidine (Fluka™).

Stability of the MenA and MenA-CDI-DMA products was assessed by using colorimetric and NMR methods to determine their average degree of polymerisation (*avDP*). Samples were incubated in glass vials in 25 mM His buffer, pH 6.5, for 96 hours at one of three temperatures (37, 49, or 57°C) and, at the end of the incubation period, the samples were stored at 4°C.

Colorimetric stability study

The chemical *avDP* is expressed by the ratio $[P_t]/[P_{me}]$, where $[P_t]$ is the total phosphorus concentration and $[P_{me}]$ is the terminal monoester phosphate concentration. $[P_t]$ was determined colorimetrically as described in ref. 132. $[P_{me}]$ was determined by measuring the inorganic phosphate P_i released by enzymatic reaction with potato acid phosphatase [133].

Figures 1 and 2 show the *avDPs* of MenA and MenA-CDI-DMA as a function of time (t).

Kinetic constants (k) of saccharide hydrolysis (seen in Figures 1 and 2 as the drop in avDP) were analysed as described in reference 134. Two distinct aspects of k were analysed:

— k as a function of $avDP$ and t in the standard kinetic equation; and then

— k as function of frequency factor (A), activation energy (ΔG_a) and temperature (T) in the Arrhenius equation.

It was assumed that hydrolysis proceeded to completion with satisfactory first-order kinetics:

$$\frac{davDP}{dt} = -kavDP \qquad\qquad avDP = avDP_0 \exp(-kt)$$

where $avDP_0 = avDP$ at $t = 0$.

The logarithmic form is:

$$\ln avDP = \ln avDP_0 - kt$$

k is defined by the slope of ln $avDP = \mathrm{f}(t)$ plot.

The Arrhenius equation indicates the correlation between the kinetic constants at various temperature values and the activation energy for the hydrolysis reaction:

$$k = A\exp(-\Delta G_a / RT)$$

$$\ln k = \ln A - \Delta G_a / RT$$

where $R = 8.314 \times 10^{-3}$ KJ/mol K.

ΔG_a is calculated from the slope of straight line obtained by plotting ln k as a function of reciprocal temperature (1/T). In this study we analysed only the total activation energy of hydrolysis reaction, without the separation of the single contributions from the activation enthalpy and activation entropy ($\Delta G_a = \Delta H_a + T\Delta S_a$).

Table 1 summarises the colorimetric *avDP* data and the kinetic constants of the hydrolysis reaction at various temperatures:

	T (K)	***avDP***					**k (s^{-1})**
		0 h	**24 h**	**48 h**	**72 h**	**96 h**	
MenA	310	21.453	17.452	14.197	11.550	9.396	2.4×10^{-6}
	322	21.453	14.028	9.173	5.998	3.922	4.9×10^{-6}
	330	21.453	10.956	5.595	2.857	1.459	7.8×10^{-6}
MenA-CDI-DMA	310	21.453	21.192	20.994	19.524	18.640	1.9×10^{-7}
	322	21.453	22.410	19.127	17.472	15.491	9.2×10^{-7}
	330	21.453	20.227	16.555	13.864	11.600	1.8×10^{-6}

Arrhenius plots of rate constants obtained at 37, 49, and 57°C, indicate that the activation energy of hydrolysis reaction in 25 mM His buffer, pH 6.5, is 50.1 KJ/mol (12.0 Kcal/mol) for MenA and 94.9 KJ/mol (22.7 Kcal/mol) for MenA-CDI-DMA. Standard errors, estimated by linear least squares regression of Arrhenius plots, are ± 5.0 KJ/mol (± 1.2 Kcal/mol) for ΔG_a values. Thus, the modified polysaccharide of the invention is nearly twice as stable as its unmodified counterpart.

NMR stability study

In order to verify the *avDP* obtained by the colorimetric method, ^{31}P NMR analytical experiments were carried out. The *avDP* data were calculated by the integration ratio between P_{me} and $P_{in\ chain}$ signals (see Figure 3).

^{1}H and ^{31}P NMR samples were prepared by dissolving lyophilized oligosaccharides in 0.75 ml of 99.9% D_2O (Aldrich™) to give 10-15 mM concentrated solutions. In all experiments, 5 mm Wilmad™ NMR tubes were used. NMR spectra were recorded at 298 K on a Bruker™ NMR Spectrometer Avance DRX 600 MHz with a BGU unit. A 5 mm TBI triple resonance probe with self shielded z-gradients was used. For processing data the Bruker XWINNMR 3.0 software was used. ^{1}H standard spectral acquisition conditions are to collect 32 k data points over a spectral window of 6000 Hz with 4 scans. ^{1}H NMR spectra were Fourier-transformed after applying a 0.1 Hz line broadening function and referenced relative to the acetate anion resonance at 1.91 ppm or that of monodeuterated water at 4.72 ppm. ^{31}P standard spectral acquisition conditions are to collect 32 k data points over a spectral window of 3000 Hz with 128 scans. 2.0 Hz line broadening function was used.

As shown in Figure 4, the colorimetric and ^{31}P NMR methods agree for all temperature values, with only a slight down-translation being evident (no effects in ln *avDP* = f(*t*)).

The results of the colorometric and ^{31}P NMR methods of analysis are summarised in Table 2:

Analytical method	T (K)	*avDP*					k (s^{-1})
		0 h	24 h	48 h	72 h	96 h	
Colorimetric determination	310	21.453	21.192	20.994	19.524	18.640	1.9×10^{-7}
	322	21.453	22.410	19.127	17.472	15.491	9.2×10^{-7}
	330	21.453	20.227	16.555	13.864	11.600	1.8×10^{-6}
^{31}P-NMR determination	310	20.407	19.573	19.170	18.450	18.253	3.3×10^{-7}
	322	20.407	16.986	14.836	12.556	10.051	2.0×10^{-6}
	330	20.407	15.984	12.123	9.860	8.079	2.7×10^{-6}

By improving the analysis by the spectral shape of P_{me}, two different molecular species are seen to arise (see Figure 3). A lower kinetic constant rate is evident for the up-field signal.

By extrapolation of acquired data at lower temperature (*e.g.* the typical storage temperature of 2 to 8°C), it is possible to extrapolate these data to a 2 year time scale (see Figure 5).

Comparing the results obtained by these investigations on the stability of MenA and MenA-CDI-DMA, a significant increase of stability of the modified product is observed. The extrapolation to a longer time scale indicates that the degradation of the MenA-CDI-DMA is sufficiently reduced to allow distribution of the product for 2 years.

Structural characterization

Figure 6 shows a ^1H NMR spectrum of a MenA-CDI-DMA sample at 298 K with indicative signal assignments. The NMR profile suggests high similarity between the MenA oligosaccharide and the MenA-CDI-DMA oligosaccharide. ^1H-methyl DMA signals are seen in the 2.6-3 ppm spectral region. The 2.73 ppm peak corresponds to the ^1H-methyl of free DMA, as a residual reagent in solution. The 2.91 ppm peak was tentatively assigned as the ^1H-methyl DMA bond to oligosaccharide chain.

The ^{31}P NMR profiles are similar to the MenA oligosaccharide. Only a short down-field shift of the P_{me} signal is observed (see Figure 3).

The modified saccharides of the invention are therefore structurally similar to their native counterparts, which should mean that antigenicity and immunogenicity are unaffected.

Competitive ELISA assays

A competitive ELISA assay was used to correlate MenA, MenA-CDI and MenA-CDI-DMA oligosaccharides with their ability to displace specific antibodies. The % of inhibition plotted as a function of competitor concentration (mg/mL) are shown in the Figure 7. All samples showed a similar behaviour, reaching about 100% of inhibition at 10^{-1} mg/mL competitor concentration.

This confirms that modification of saccharides using the invention does not result in loss of antigenicity.

Conjugation

The modified MenA saccharide (MenA-CDI-DMA) was conjugated to CRM_{197} protein by the process summarised in Figure 8. The basic steps in the conjugation process are:

- hydrolysis of MenA polysaccharide to give oligosaccharide fragments
- sizing of the oligosaccharide fragments
- reductive amination of terminal aldehyde groups on the sized oligosaccharides
- protection of terminal $-NH_2$ groups by Fmoc group before the CDI reaction
- intrinsic de-protection of $-NH_2$ groups during the DMA reaction
- activation of terminal $-NH_2$ groups by SIDEA (N-hydroxysuccinimide adipic acid)
- covalent attachment to CRM_{197} protein

a) Hydrolysis

MenA polysaccharide was hydrolysed in 50 mM sodium acetate buffer, pH 4.75 for about 3 hours at 73°C. Hydrolysis was controlled in order to obtain oligosaccharides with an average degree of polymerisation (DP) of approximately 15, as determined by the (w/w) ratio between the total organic phosphorous and the monoester phosphate.

b) Sizing

This step selects a defined population of oligosaccharides generated during the hydrolysis process. The hydrolysate obtained above was ultrafiltered through a 30kDa cut-off membrane (12 diafiltration volumes of 5 mM acetate buffer, pH 6.5) to remove the long-length chains in the retentate moiety.

c) Introduction of a primary amino group at the reducing terminus

Ammonium acetate was added to the sized oligosaccharide solution for a final concentration of 300 g/L, and sodium cyano-borohydride was added to a final concentration of 73 g/L. After adjusting the pH to 6.5±0.2, the mixture was incubated at 37°C for 5 days.

The amino-oligosaccharides were then purified by ultrafiltration through a 3kDa cut-off membrane using 13 volumes of 0.5 M NaCl. This step removes short-length saccharide chains (DP<6-7) giving a final degree of average polymerisation of ~15.

The retentate moiety was diafiltered with 4 volumes of 10mM TAB (tetrabutyl ammonium bromide) and then with 7 volumes of H_2O to exchange Na^+ to TAB^+. The positive organic ion improves the saccharide's solubility in DMSO (required for the next derivatisation steps) to about 10 g/L.

Purified oligosaccharides were dried with a rotary evaporator to remove water and then were solubilized in DMSO solvent at concentration of about 10g/L.

The purified amino-oligosaccharide solution was analysed for phosphorous content by the procedure of Chen [132] and for the amount of introduced amino groups by the procedure of Habbeb [135].

As an alternative to ultrafiltration to remove short-chain saccharides, a Q-Sepharose Fast Flow column was used, but a further exchange process onto a SP-Sepharose (Pharmacia™) column is then needed to effect the Na^+/TAB^+ conversion.

d) Protection of terminal amino group with Fmoc reagent

The amino-oligosaccharides were reacted with Fmoc-OSu (N-9-Fluorenylmethoxycarbonyloxy) (Sigma) according to the molar ratio $-NH_2$:Fmoc-OSu = 1:20. The mixture was incubated overnight under stirring at room temperature and was precipitated with acetone (80% v/v final concentration). The precipitate was collected by centrifugation and washed several times with acetone to remove unreacted Fmoc-OSu reagent.

e) Stabilizing reaction with CDI and DMA reagents

The protected amino-oligosaccharides were solubilised in DMSO at 10g/L and added to CDI at a molar ratio of CDI:total phosphorous = 20:1. The mixture was incubated overnight under stirring at room temperature and was precipitated with acetone (80% v/v final concentration). The precipitate was collected by centrifugation and washed several times with acetone to remove unreacted CDI reagent.

The product obtained above was solubilised in DMSO at 10g/L and added to a solution of DMA in ethanol (about 5.6 M) according to the molar ratio of DMA:total phosphorous = 20:1. The mixture was incubated overnight under stirring at room temperature and was precipitated with acetone (80% v/v final concentration). The precipitate was collected by centrifugation and washed several times with acetone to remove unreacted DMA.

The purified oligosaccharides were then dried under vacuum to remove traces of organic solvents.

f) Chromatographic ionic exchange

The dried oligosaccharide was solubilised in water at 10 g/L and loaded onto a SP-Sepharose Fast Flow (Pharmacia™) column equilibrated in 1 M NaCl, in order to perform the Tab^+/Na^+ exchange. The column was then washed with 5 column volumes (CV) of water to recuperate traces of product adsorbed to resin. The oligosaccharide was then dried with rotary evaporator to remove water.

g) Derivatisation to active ester

The dried product was solubilised in water at a 40 mM amino group concentration, then 9 volumes of DMSO were added followed by TEA (triethyl-amine) at a final concentration of 200 mM. To the resulting solution, adipic acid N-hydroxysuccinimido diester (SIDEA) was added for a final concentration of 480 mM.

The reaction was maintained under stirring at room temperature for 2 hours, then the activated oligosaccharide was precipitated with acetone (80% v/v final concentration). The precipitate was collected by centrifugation and washed several times with acetone to remove unreacted SIDEA.

The purified oligosaccharides were then dried under vacuum to remove the solvent.

The amount of active ester groups introduced into the oligosaccharide structure was determined by a colorimetric method as described in reference 136.

The activated oligosaccharide was analysed by ^1H NMR as described above to confirm the chemical modifications. The proton NMR profiles established in previous experiments were used to evaluate several lots of product. The saccharide signals were assigned by inspection of 1D (Figure 9) and 2D hetero-correlate (^1H,^{13}C; Figures 10A & 10B) NMR spectra and were shown to be characteristic of the modified MenA.

About 5 mg of each sample were dissolved in 750μL D_2O and the spectra were recorded on a Bruker Avance 600 MHz spectrometer.

Inspection of the ratio of CH_3^{DMA} groups and $H_1^{saccharide\ ring}$ provided a stabilizing reaction yield of between 70% and 75 %.

The modified saccharide proton profile is maintained, and substantial modifications concerning the O-acetyl status and the structural conformation are not evident from the NMR analysis. However, the carbamate groups change the local magnetic field and thus the assignment of ^1H NMR spectrum is complicated.

In Figure 11, superimposed spectra of modified and native MenA oligosaccharides are shown. A down-field shift of H_3, H_4 and H_2 signals is evident because the carbamate groups in C_3 and C_4 ring position are nearer than other nuclei, as for instance H_1. Other shifts are suggested from the spectra but their assignment is not completely certain.

Figure 12 shows that chemical derivatisation does not cause any change in the ^{31}P NMR spectrum.

h) Conjugation to CRM_{197}

The dried activated oligosaccharide was added to a 45 mg/mL solution of CRM_{197} in 10 mM phosphate buffer, pH 7.2, according to a molar ratio of ester groups:protein = 12:1. The reaction was maintained under stirring at room temperature overnight and the obtained conjugate was purified by tangential ultrafiltration through 30kDa cut-off membrane using 50 volumes of phosphate buffer 10 mM, pH 7.2. The product was sterile filtered and stored at –20°C until vaccine formulation.

The purified conjugate was analysed for protein content (microBCA Protein Assay), saccharide content (colorimetric determination of phosphorous), free saccharide content (chromatographic analysis), HPLC profile (on TSKgel G4000SWXL 7.5 mm IDx30cm), NMR profile and SDS-PAGE.

Time-dependent stability of the conjugate

The stability of the CRM_{197} conjugate of the MenA-CDI-DMA oligosaccharide was assessed by monitoring the appearance of free saccharide in solution, due to hydrolysis, during four weeks of storage at 37°C, in comparison to a CRM197 conjugate of unmodified MenA oligosaccharide.

Free (*i.e.* non-conjugated) saccharide was determined uding reversed-phase chromatography on ISOLUTE™ C4 cartridge column (IST™) to isolate the non-conjugated chains, and then by permeated saccharide with HPAE-PAD chromatography.

Total saccharide (*i.e.* both conjugated and non-conjugated) was determined by a method for the quantitative analysis of N-acetyl mannosammine phosphate, which uses high-performance anion-exchange chromatography with pulsed-amperometric detection (HPAE-PAD) [137].

The ratio of unconjugated saccharide to total saccharide was expressed as a percentage (%FS).

In a first experiment, %FS developed as follows (Figure 13):

Time (days)	0	7	14	21	28
Modified	16.8	14.7	23.8	21.7	23.3
Natural	11.0	-	27.0	36.6	39.5

In a second experiment, results were as follows (Figure 14):

Time (days)	0	7	14	21	28
Modified	1.8	1.5	4.2	4.9	8.2
Natural	4.8	5.5	19.3	22.7	28.3

The modified MenA oligosaccharide conjugate is clearly much more resistant to hydrolysis than its natural counterpart at elevated temperatures. After 28 days at 37°C, for instance, the percentage of released saccharide is 6.4 % for the modified oligosaccharide *vs.* 23.5 % for the natural sugar.

In further work to test lot-to-lot consistency using the modified MenA saccharide, the appearance of free saccharide from conjugates was monitored for 8 weeks at 37°C. Results for three lots were:

Time (days)	0	7	14	28	56
Lot A	1.7.	2.9	3.2	5.8	8.7
Lot B	1.0	4.2	4.5	6.5	10.9
Lot C	2.2	4.5	5.4	8.2	11.4

The modified conjugate is thus stable over an extended period. A free saccharide level of less than 12% is well within acceptable limits, even at above-normal temperature.

pH-dependent stability of the conjugate

Stability of the conjugates of modified and unmodified MenA oligosaccharides was tested by monitoring the appearance of free saccharide at different pH between 6.0 and 8.0 after being stored at 37°C for 28 days. Modified (Lot 5) and unmodified (Lot RS040101) oligosaccharides were compared and the increases in free saccharide (Ä%FS) between days 0 and 28 were as follows (Figure 15):

pH *	6.0	6.5	7.0	7.5	8.0
Modified	10.3	5.4	8.9	8.3	26.1
Native	43.5	30.1	36.1	20.3	30.5

* pH±0.1

The modified MenA conjugate thus shows a much lower hydrolysis reaction rate than conjugate of the native MenA oligosaccharide in the pH range 6.5-7.5. At pH 8.0, where carbamate stabilising groups are removed, the effect is less marked.

Immunogenicity of modified conjugates

Purified CRM_{197} conjugates of the modified MenA oligosaccharide were used to immunise mice in order to verify that the modification does not remove the saccharide's immunogenicity.

The vaccine was formulated to give a single human dose (SHD) of 10μg saccharide in a 0.5ml volume. Two formulations were made: a liquid formulation and a lyophilized formulation. Both contain an aluminium phosphate adjuvant at 0.6mg Al^{3+}/ml in the final dosage form, with an aqueous suspension of the adjuvant being used to reconstitute the lyophilised formulation.

The liquid formulation of the modified oligosaccharide conjugate was compared to the lyophilised formulation of the native (*i.e.* unmodified) oligosaccharide conjugate.

Mice were immunised with 1/5 of the SHD, the vaccines being diluted with saline before each immunization. 10 Balb/c mice (female, 6-8 weeks old) per immunisation group were injected subcutaneously with 0.5 ml of the vaccine at time zero and then four weeks later. Bleedings were performed before the first immunisation and at week 6 (pre and post-II sera), with sera being stored at -70°C.

<u>Anti-polysaccharide titres</u>

Specific anti-MenA polysaccharide total IgG antibodies were determined in the sera of immunized animals according to the CDC procedure for MenA human sera analysis [138], adapted for animal sera analysis, with some minor changes.

Each individual mouse serum was analyzed in duplicate by a titration curve. GMT was calculated for immunization groups. Anti-MenA polysaccharide titre was expressed in Mouse Elisa Units (MEU), with software based on the Reference Line Assay Method being used for MEU calculation.

IgG subclasses analysis was performed with pooled post-II sera of the immunization groups, using alkaline phosphatase-anti mouse IgG1, or IgG2a, or IgG2b or IgG3 (Zymed) as secondary conjugate in the ELISA procedure. Titres were expressed as OD_{405nm} obtained at a dilution 1:3200 of the pooled post-II sera after 30 minutes of substrate development.

Figure 16 shows anti-MenA-pS IgG titres (GMT) induced by the two lots of MenA modified conjugate (lots 3 and 5) compared to the lyophilised unmodified MenA conjugate (lot 002011) using aluminium phosphate adjuvant. Both modified conjugates induced a titre very similar to that induced by the unmodified MenA conjugate.

Figure 17 shows IgG subclasses analysis of the pooled sera (diluted 1:3200) from immunisation with modified and unmodified MenA conjugates. The most represented subclass in all sera is IgG1, the subclass predominantly induced in mice by T-dependent antigens when presented as proteins. Because the MenA capsular saccharide is naturally a T-independent antigen which is not able to induce immunological memory, this shows that the conjugation achieves its purpose.

Anti-MenA pS titre specificity was determined by a competitive ELISA using MenA pS as competitor at a final concentration of 25µg/ml. As shown in Figure 18, there is very good inhibition of the titre induced by the modified and unmodified conjugates, indicating that all conjugates were able to induce anti-MenA pS-specific titres.

Serum bactericidal assay (SBA) against *N.meningitidis* serogroup A

The functionality of antibodies induced by immunisation with the conjugates was analyzed in an *in vitro* bactericidal assay to measure complement-mediated lysis of bacteria.

Pooled post-II sera for each immunisation group were used. They were inactivated for 30 minutes at 56°C before the use in the assay. 25% baby rabbit complement was used as the source of complement (Pel Freeze). The bactericidal titre was expressed as the reciprocal serum dilution yielding 50% killing of the bacteria. Activity against two serogroup A strains was tested: F8238 & F6124.

Titres were as follows:

Target strain	Lot 3	Lot 5	Lot 002011
F8238	2048-4096	2048	4096-8192
F6124	4096	2048	4096

Therefore all three conjugates induce good bactericidal titres against both strains, and the titres induced by the modified oligosaccharides are not significantly lower than those obtained using the native sugar structures. Advantageously, however, the modified oligosaccharides are significantly more stable than the native oligosaccharides. The invention therefore provides antigens which retain the immunogenic potential of the native MenA capsular saccharide, but which offer improved resistance to hydrolysis during storage.

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

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CLAIMS

1. A modified capsular saccharide comprising a blocking group at a hydroxyl group position on at least one of the monosaccharide units of the corresponding native capsular saccharide.

2. The modified capsular saccharide of claim 1 wherein the at least one monosaccharide unit is a non-terminal monosaccharide unit.

3. The modified capsular saccharide of claim 1 or 2 which comprises at least one free hydroxyl group or amino group.

4. The modified capsular saccharide of claim 3 wherein the at least one free hydroxyl group is a terminal anomeric hydroxyl group.

5. The modified capsular saccharide of claim 1 wherein the blocking group is an electron-withdrawing group.

6. The modified capsular saccharide of claim 1 or claim 2 wherein the blocking group is of the formula:

$$\text{-O-X-Y} \qquad \text{or} \qquad \text{-OR}^3$$

wherein

X is C(O), S(O) or SO_2;

Y is C_{1-12} alkyl, C_{1-12} alkoxy, C_{3-12} cycloalkyl, C_{5-12} aryl or C_{5-12} aryl-C_{1-6} alkyl, each of which may optionally be substituted with 1, 2 or 3 groups independently selected from F, Cl, Br, CO_2H, $CO_2(C_{1-6}$ alkyl), CN, CF_3 or CCl_3; or Y is NR^1R^2;

R^1 and R^2 are independently selected from H, C_{1-12} alkyl, C_{3-12} cycloalkyl, C_{5-12} aryl, C_{5-12} aryl-C_{1-6} alkyl; or R^1 and R^2 may be joined to form a C_{3-12} saturated heterocyclic group;

R^3 is C_{1-12} alkyl or C_{3-12} cycloalkyl, each of which may optionally be substituted with 1, 2 or 3 groups independently selected from F, Cl, Br, $CO_2(C_{1-6}$ alkyl), CN, CF_3 or CCl_3; or R^3 is C_{5-12} aryl or C_{5-12} aryl-C_{1-6} alkyl, each of which may optionally be substituted with 1, 2, 3, 4 or 5 groups selected from F, Cl, Br, CO_2H, $CO_2(C_{1-6}$ alkyl), CN, CF_3 or CCl_3;

7. The modified capsular saccharide of claim 3 wherein the blocking group is -OC(O)NR^1R^2 or -OC(O)CF_3.

8. The modified capsular saccharide of claim 4 wherein the blocking group is $-OC(O)NR^1R^2$, and R^1 and R^2 are independently selected from C_{1-6} alkyl.

9. The modified capsular saccharide of claim 5 wherein R^1 and R^2 are both methyl.

10. The modified capsular saccharide of any preceding claim wherein at least 10% of the monosaccharide units comprises a blocking group.

11. The modified capsular saccharide of any preceding claim, wherein the corresponding capsular saccharide comprises monosaccharide units linked by phosphodiester bonds.

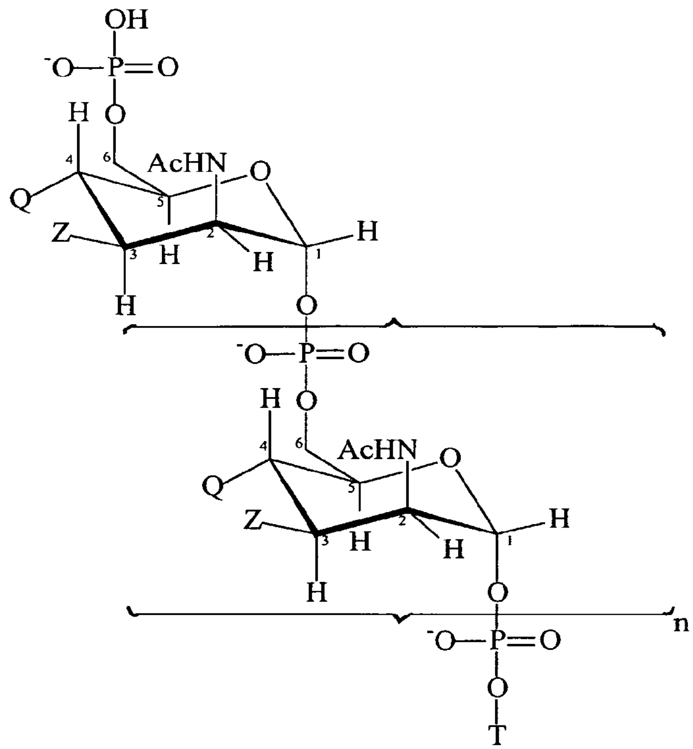
12. The modified capsular saccharide of claim 11, wherein the corresponding capsular saccharide is a *Neisseria meningitidis* serogroup A saccharide.

13. The modified capsular saccharide of claim 12 wherein the blocking group is at any of the 4- and/or 3-positions of the corresponding *Neisseria meningitidis* serogroup A saccharide.

14. The modified capsular saccharide of claim 12 wherein the blocking group is at any of the 4-positions of the corresponding *Neisseria meningitidis* serogroup A saccharide.

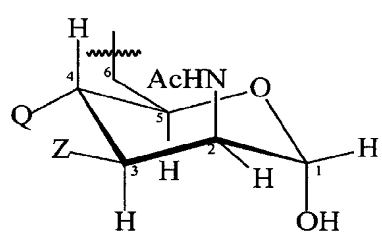
15. The modified capsular saccharide of claims 1 to 14 which is an oligosaccharide.

16. A saccharide of the formula:

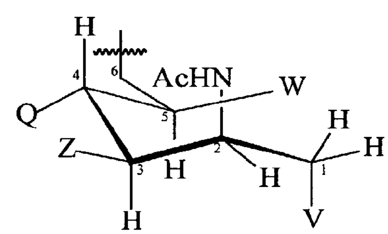


wherein

T is of the formula (A) or (B):



(A)



(B)

n is an integer from 1 to 100;

each Z group is independently selected from OH or a blocking group as defined in claims 5 to 9; and

each Q group is independently selected from OH or a blocking group as defined in claims 5 to 9;

W is selected from OH or a blocking group as defined in claims 5 to 9;

V is selected from $-NH_2$ or $-NH-E$, where E is a nitrogen protecting group;

and wherein more than about 7% of the Q groups are blocking groups.

17. The saccharide of claim 16 wherein at least 50% of the Z groups are OAc.

18. The saccharide of claims 16 or 17 wherein n is an integer from 15 to 25.

19. The saccharide of claims 16 to 18 wherein at least 10% of the Q groups are blocking groups.

20. A process for modifying a capsular saccharide comprising the steps of:

(a) providing a capsular saccharide having at least one hydroxyl group on a monosaccharide unit; and

(b) converting said at least one hydroxyl group into a blocking group.

21. The process of claim 20 wherein the blocking group is as defined in any of claims 5 to 9.

22. The process of claims 20 or 21 wherein the blocking group is $-OC(O)NR^1R^2$ and step (b) comprises the steps of:

(b1) reacting the capsular saccharide with a bifunctional reagent in an organic solvent; and

(b2) reacting the product of step (b1) with an amino compound of formula (I):

$$HNR^1R^2 \qquad (I)$$

wherein R^1 and R^2 are as defined in any of claims 3, 5 and 6.

23. The process of claim 22, wherein the organic solvent is an aprotic solvent.

24. The process of claim 23 wherein the aprotic solvent is selected from dimethylsulfoxide (DMSO), dimethylformamide (DMF), formamide, hexamethylphosphoramide (HMPA), hexamethylphosphorus triamide (HMPT), 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU) or dimethylacetamide (DMAC).

25. The process of claims 23 or 24 wherein the aprotic solvent is DMSO.

26. The process of claims 22 to 25 wherein the bifunctional reagent is selected from 1,1'-carbonyldiimidazole (CDI), carbonyl di-1,2,4-triazole (CDT), carbonyl di-1,2,3-benzotriazole (CDB), diphenylcarbonate, cyanogen bromide, phosgene or triphosgene.

27. The process of claim 26 wherein the bifunctional reagent is CDI.

28. The process of claims 20 to 27 wherein the modified capsular saccharide is a modified capsular oligosaccharide.

29. The process of claim 28 wherein the capsular saccharide in step (a) is a capsular oligosaccharide obtainable by sizing the corresponding native capsular polysaccharide.

30. The process of claim 28 wherein the capsular saccharide in step (a) is a native capsular polysaccharide and the process further comprises a step (c) in which the product of step (b) is sized, thereby providing a modified capsular oligosaccharide.

31. A process for modifying a *Neisseria meningitidis* serogroup A polysaccharide comprising the steps of:

(a) providing a native *Neisseria meningitidis* serogroup A polysaccharide;

(b) sizing said polysaccharide to provide an oligosaccharide; and

(c) converting at least one hydroxyl group of the oligosaccharide into a blocking group, in accordance with any of claims 21 to 27.

32. A process for modifying a *Neisseria meningitidis* serogroup A polysaccharide comprising the steps of:

(a) providing a native *Neisseria meningitidis* serogroup A polysaccharide;

(b) converting at least one hydroxyl group of the polysaccharide into a blocking group, in accordance with any of claims 21 to 27; and

(c) sizing the resulting polysaccharide.

33. A process for preparing the modified capsular saccharide of claims 1 to 19 which is a total synthesis process comprising forming glycosidic linkages between two or more monosaccharide units.

34. A modified capsular saccharide obtainable by the process of any of claims 20 to 33.

35. A modified capsular saccharide obtained by the process of any of claims 20 to 33.

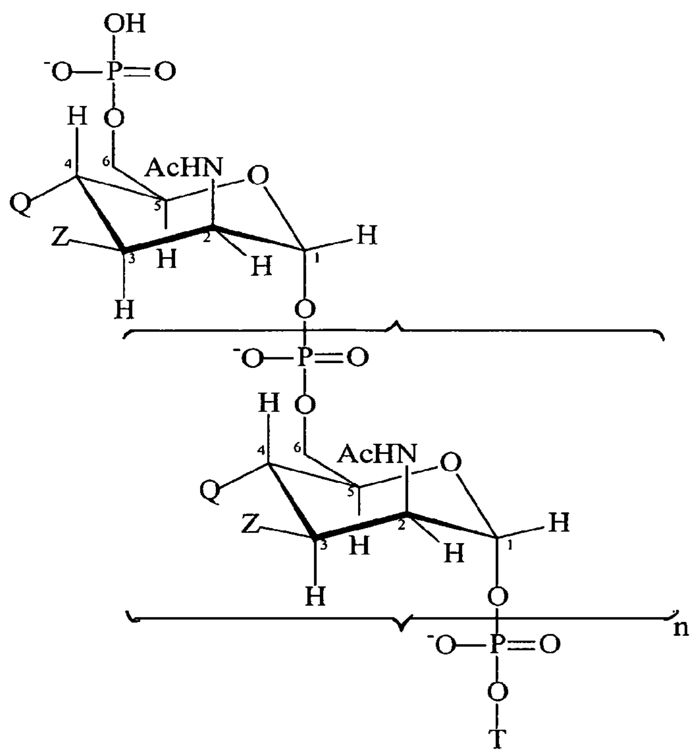
36. A saccharide-protein conjugate of a modified saccharide according to any one of claims 1 to 19, 34 or 35.

37. The conjugate of claim 36, wherein the protein is a bacterial toxin or toxoid.

38. The conjugate of claim 37, wherein the bacterial toxin or toxoid is diphtheria toxin or toxoid

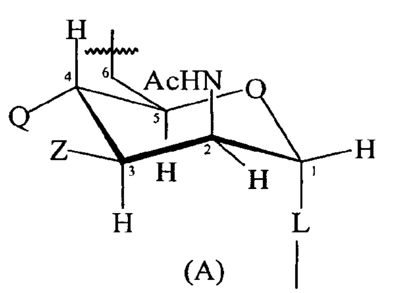
39. The conjugate of claim 37, wherein the bacterial toxin or toxoid is CRM_{197}.

40. A molecule comprising a saccharide moiety of formula:

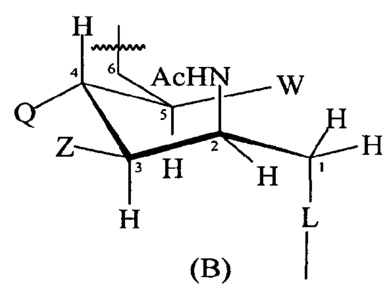


wherein

T is of the formula (A) or (B):



(A)



(B)

n is an integer from 1 to 100;

each Z group is independently selected from OH or a blocking group as defined in claims 5 to 9; and

each Q group is independently selected from OH or a blocking group as defined in claims 5 to 9;

W is selected from OH or a blocking group as defined in claims 5 to 9;

V is selected from $-NH_2$ or $-NH-E$, where E is a nitrogen protecting group;

L is O, NH, NE, S or Se;

and wherein more than about 7% of the Q groups are blocking groups.

41. A pharmaceutical composition comprising (a) a modified saccharide according to any one of claims 1 to 19, 34 or 35 and/or a saccharide-protein conjugate according to any one of claims 36 to 39 and/or a molecule according to claim 40, and (b) a pharmaceutically acceptable carrier.

42. The composition of claim 41, further comprising a saccharide antigen from one or more of serogroups C, W135 and Y of *N.meningitidis*, the saccharide optionally being an oligosaccharide and optionally being conjugated to a carrier protein.

43. The composition of claim 41 or claim 42, further comprising a vaccine adjuvant.

44. The composition of claim 43, wherein the adjuvant is an aluminium phosphate.

45. The composition of any one of claims 41 to 44, which is a vaccine against a disease caused by Neisseria meningitidis.

46. A method for raising an antibody response in a mammal, comprising administering the pharmaceutical composition of any one of claims 41 to 45 to the mammal.

47. The modified saccharide of any one of claims 1 to 18 for use as a medicament.

48. The conjugate of any one of claims 36 to 39 for use as a medicament.

49. The molecule of claim 40 for use as a medicament.

50. The use of the modified polysaccharide of any one of claims 1 to 19, 34 or 35, or the conjugate of any one of claims 36 to 39, or the molecule according to claim 40, in the manufacture of a medicament for preventing or treating a disease caused by one or more capsulate bacteria.

51. The use of claim 50, wherein the disease is bacterial meningitis.

Figure 1

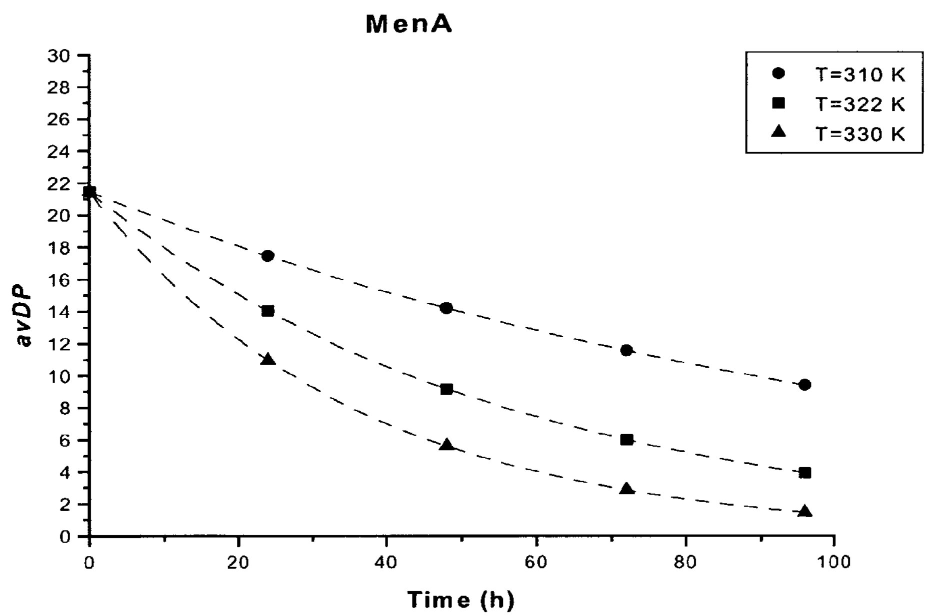


Figure 2

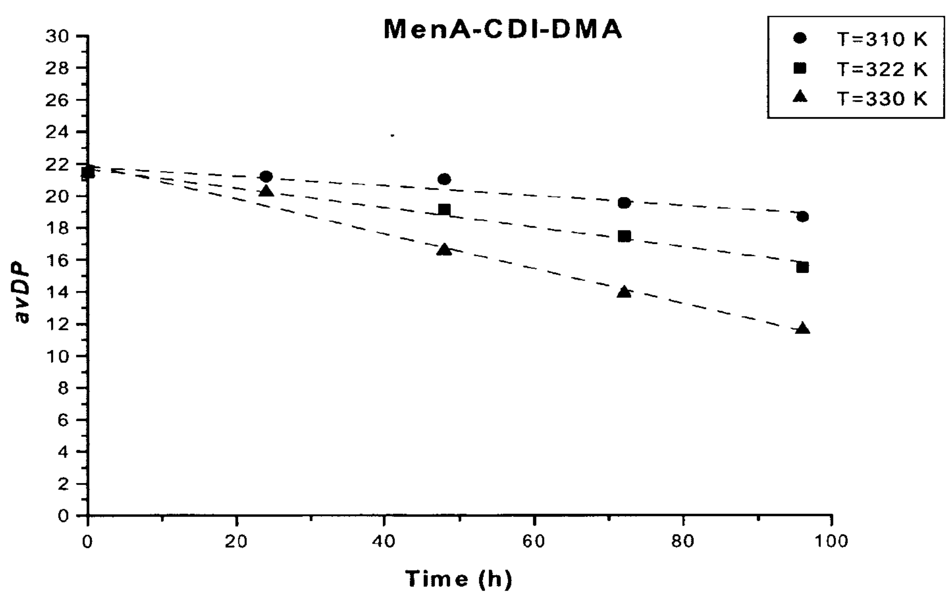


Figure 3

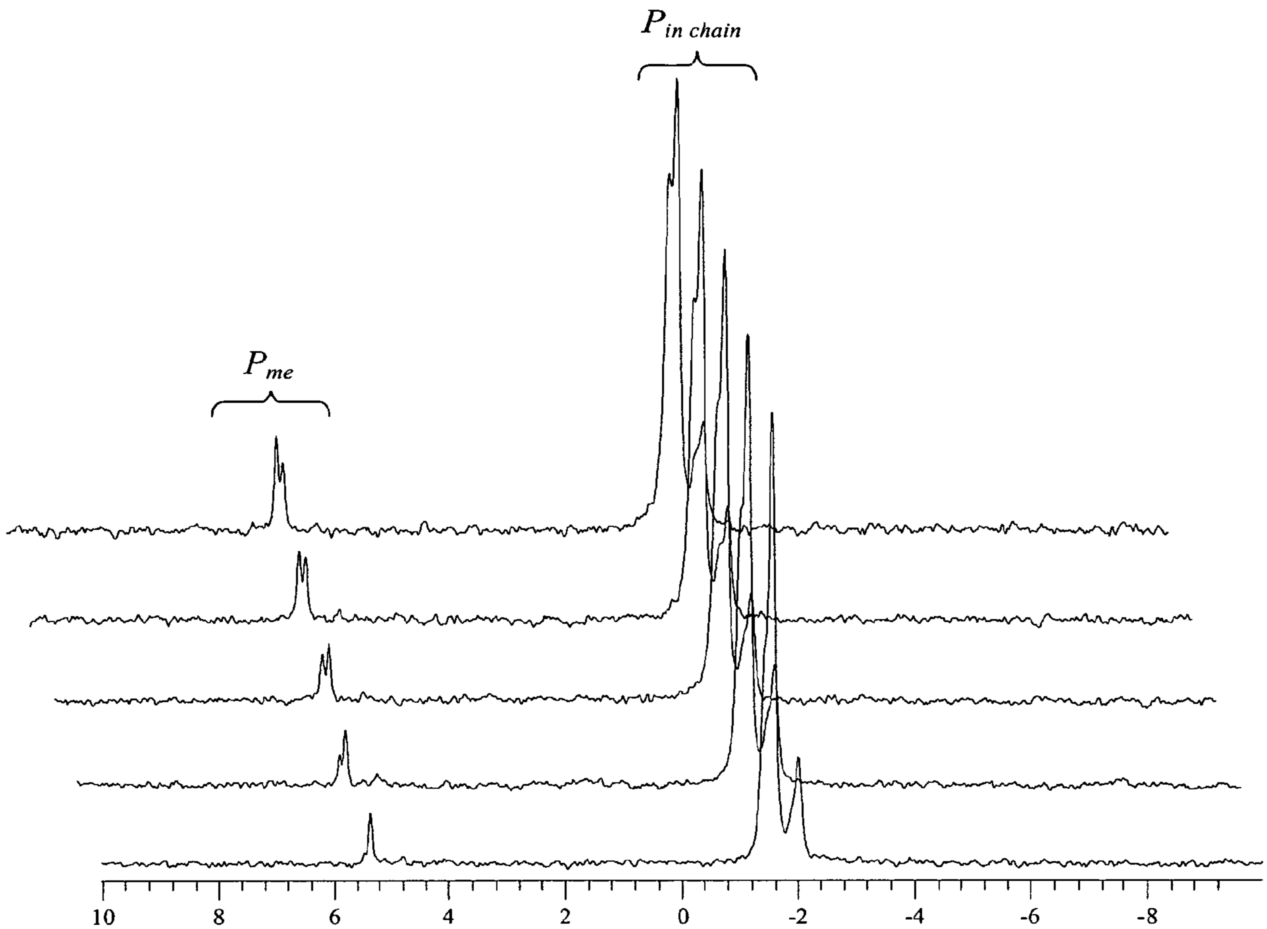


Figure 4

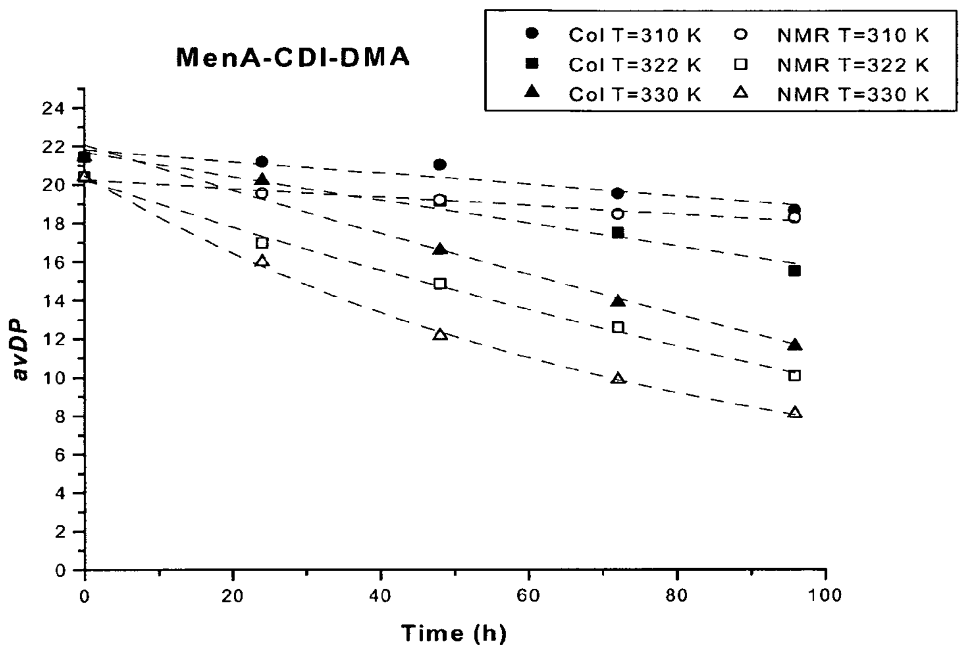


Figure 5

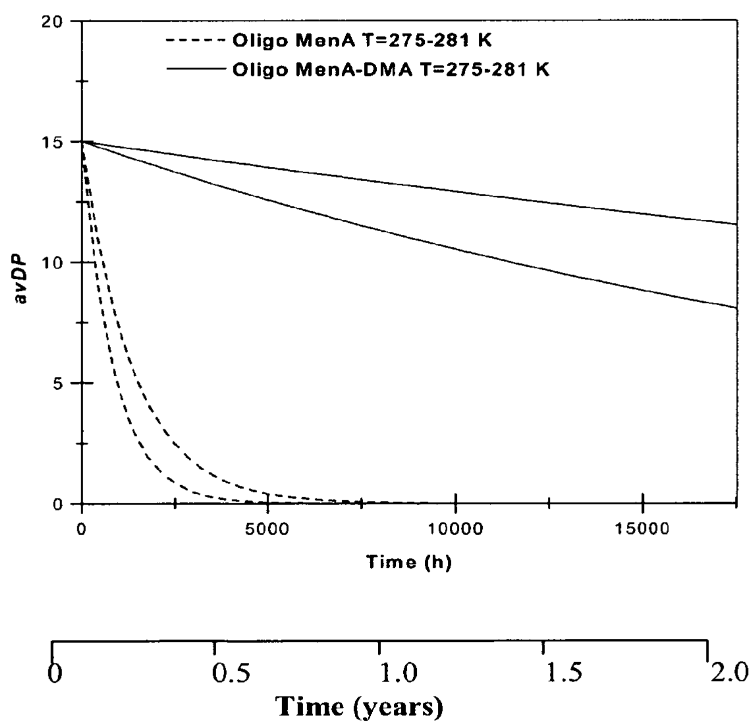


Figure 6

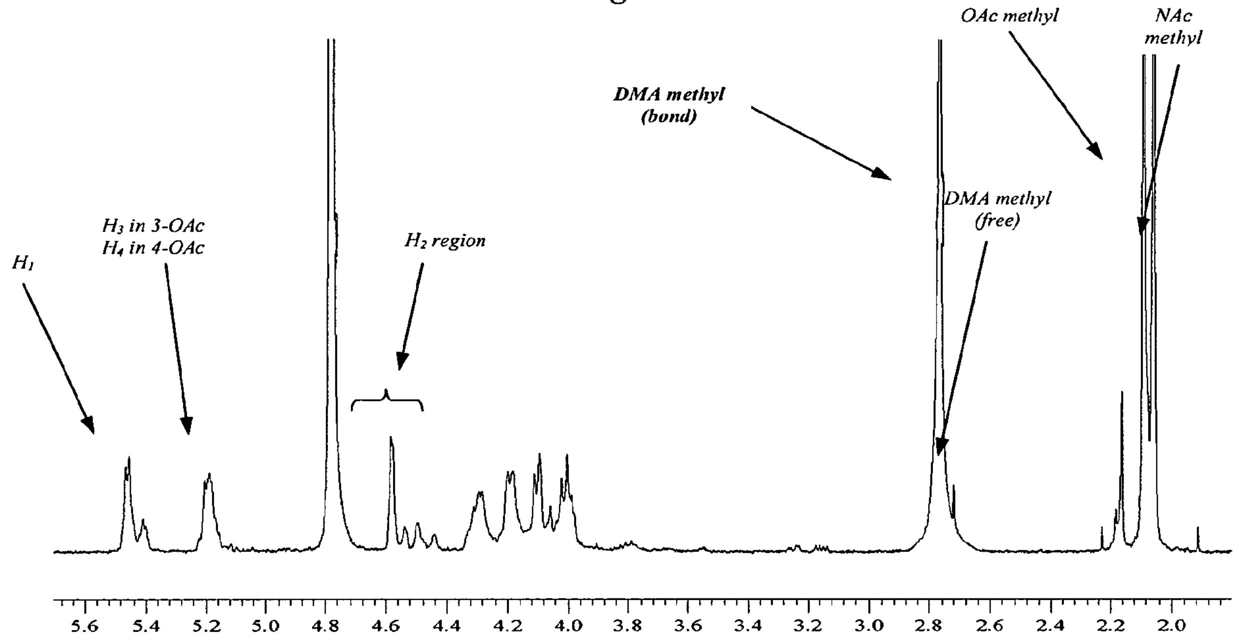


Figure 7

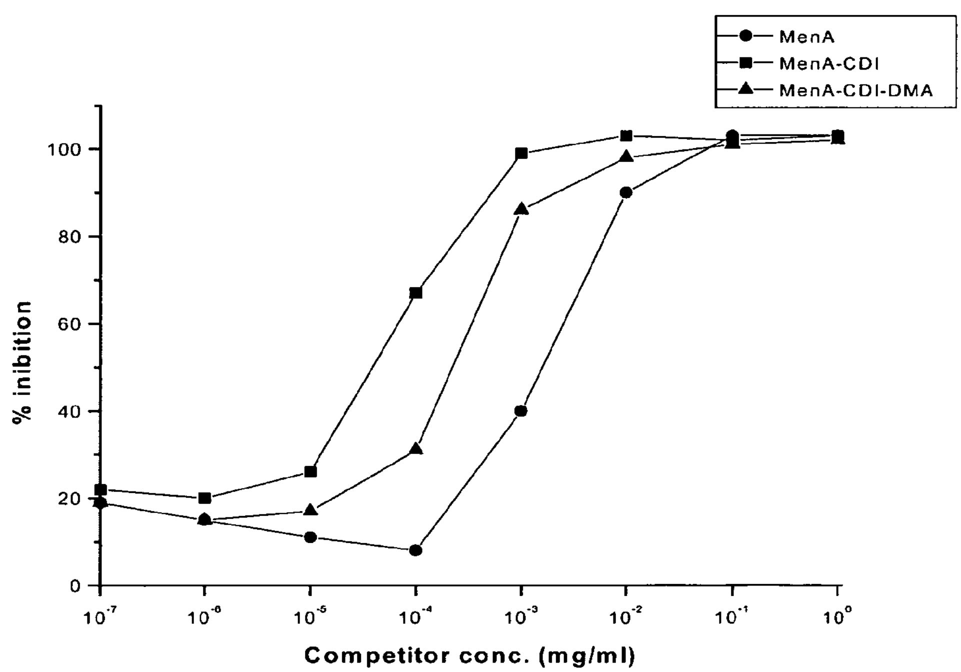


Figure 8

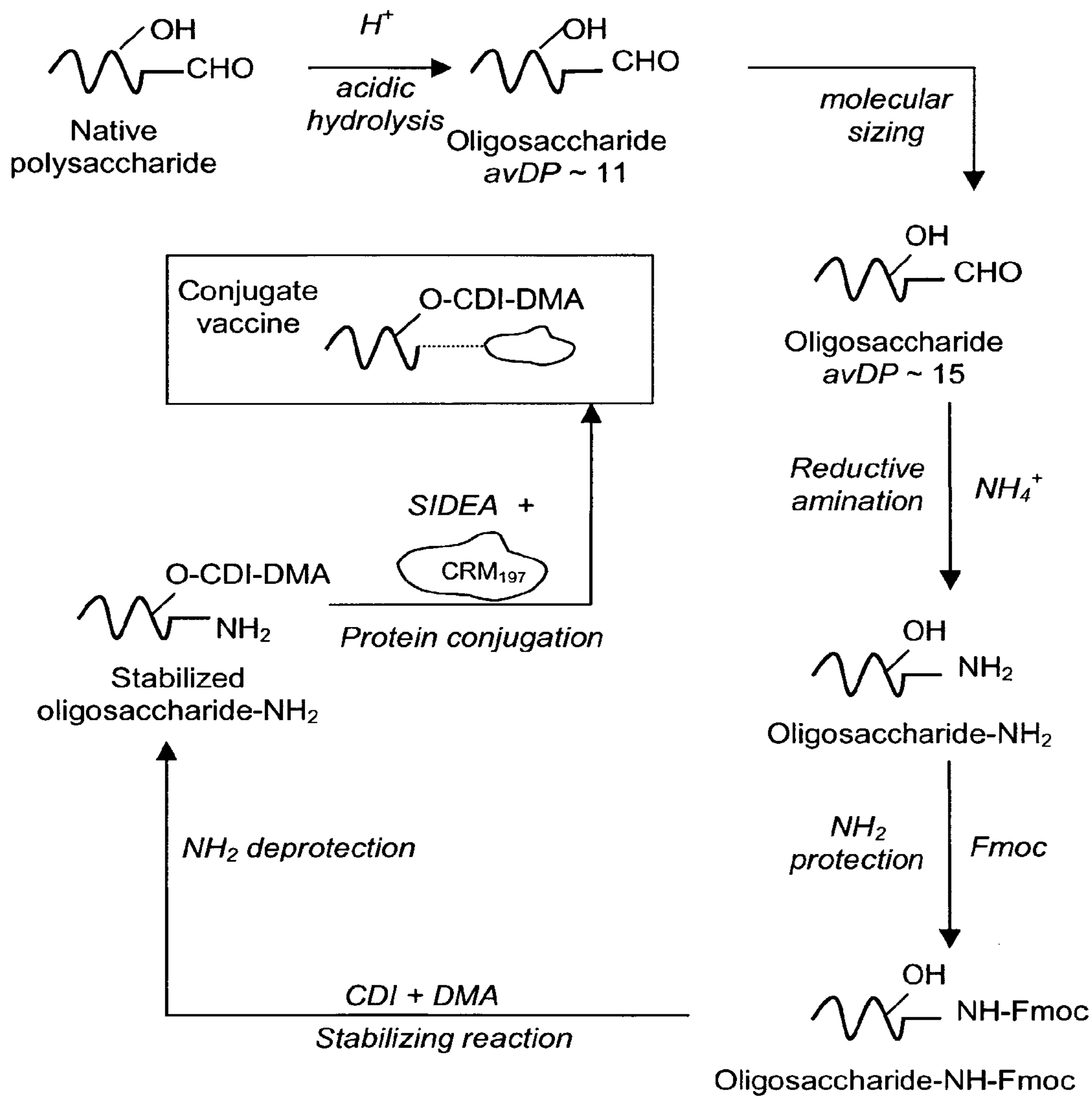

OH
CHO
Native
polysaccharide
H+
acidic
hydrolysis
OH
CHO
Oligosaccharide
avDP ~ 11
molecular
sizing
OH
CHO
Oligosaccharide
avDP ~ 15
Reductive
amination
NH4+
OH
NH2
Oligosaccharide-NH2
NH2
protection
Fmoc
OH
NH-Fmoc
Oligosaccharide-NH-Fmoc
CDI + DMA
Stabilizing reaction
NH2 deprotection
O-CDI-DMA
NH2
Stabilized
oligosaccharide-NH2
SIDEA +
CRM197
Protein conjugation
Conjugate
vaccine
O-CDI-DMA

Figure 9

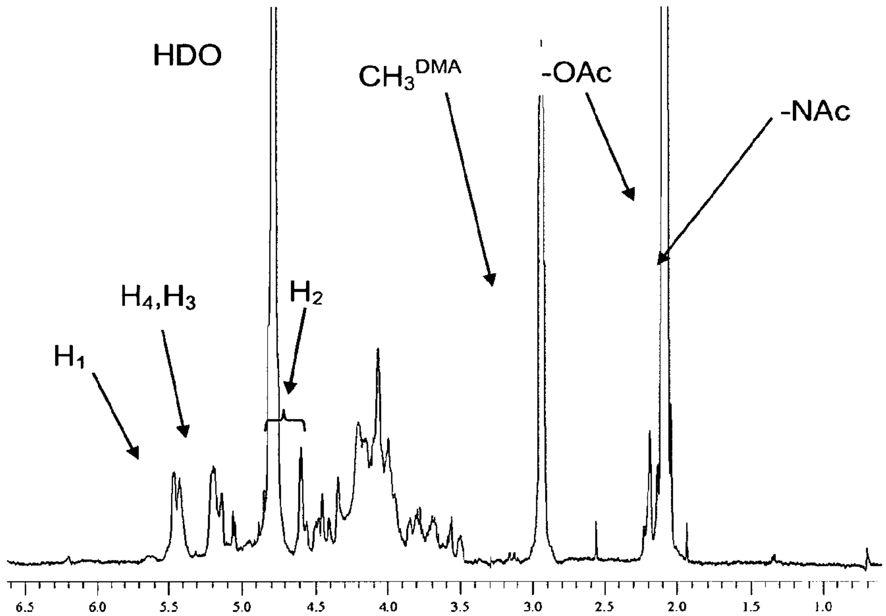


Figure 11

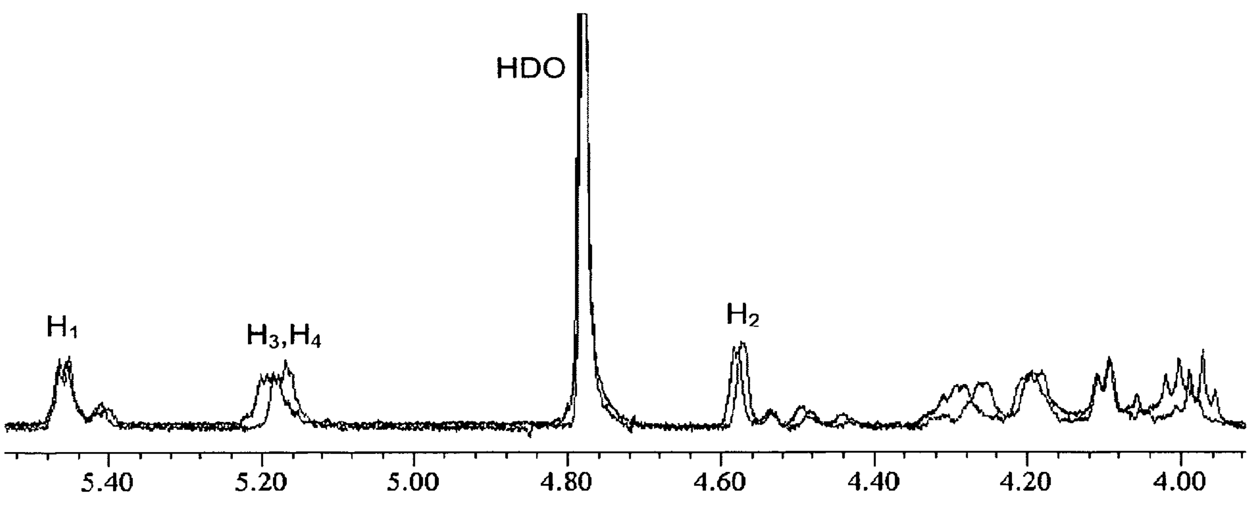


Figure 10A

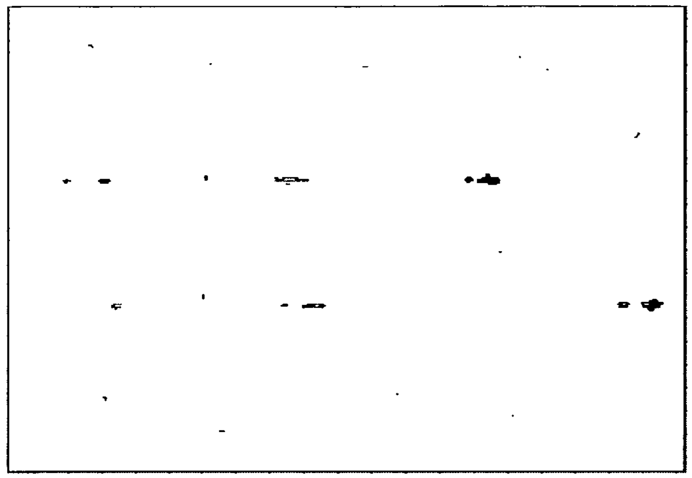


Figure 10B

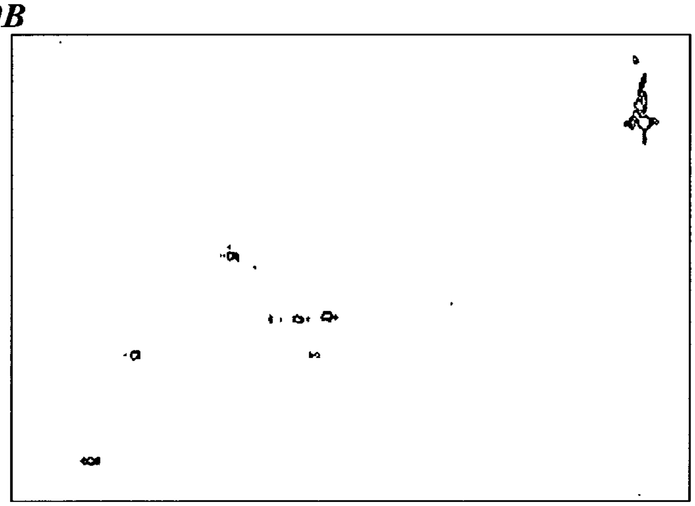


Figure 12

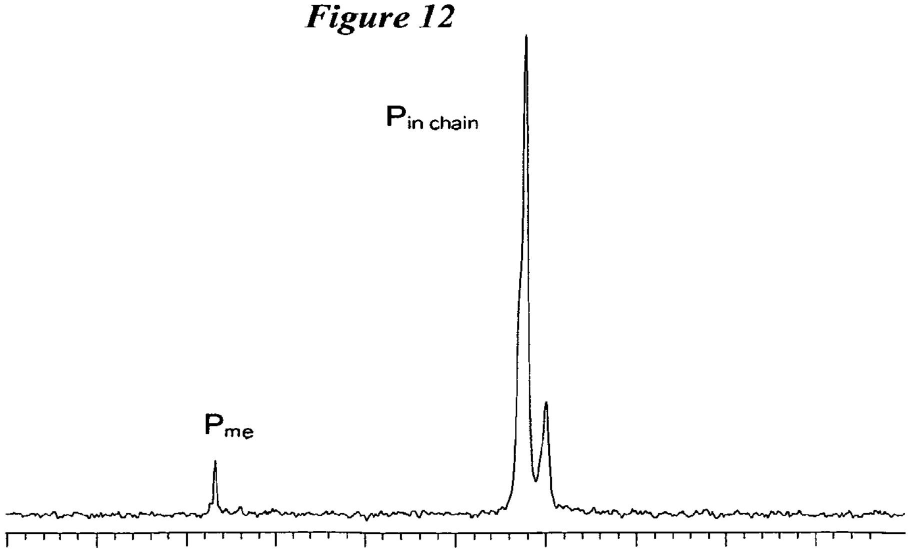


Figure 13

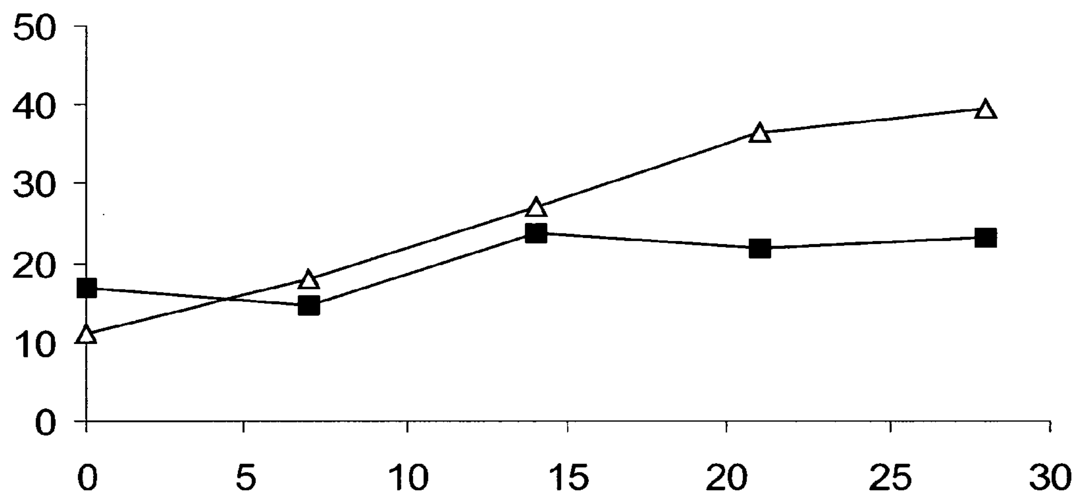


Figure 14

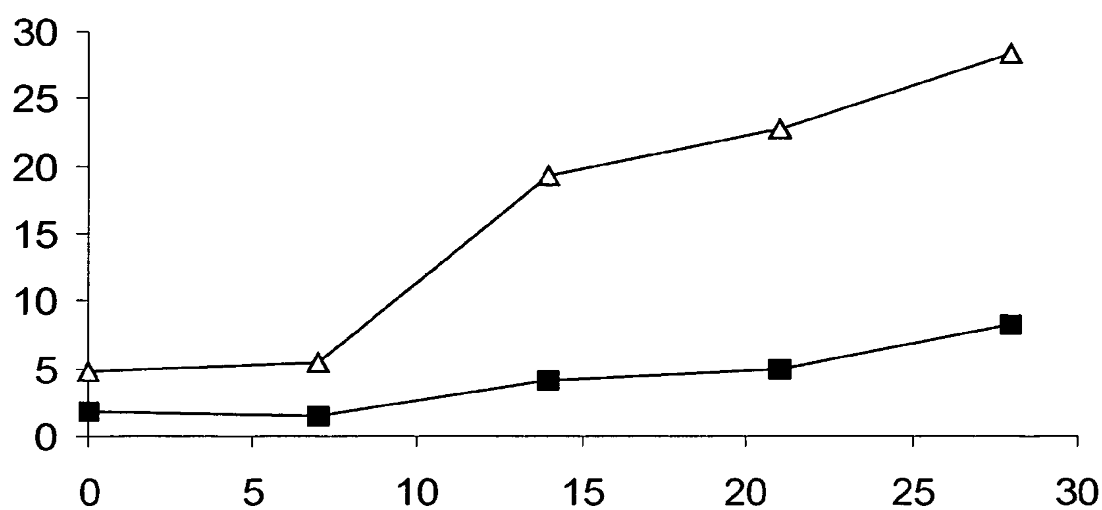


Figure 15

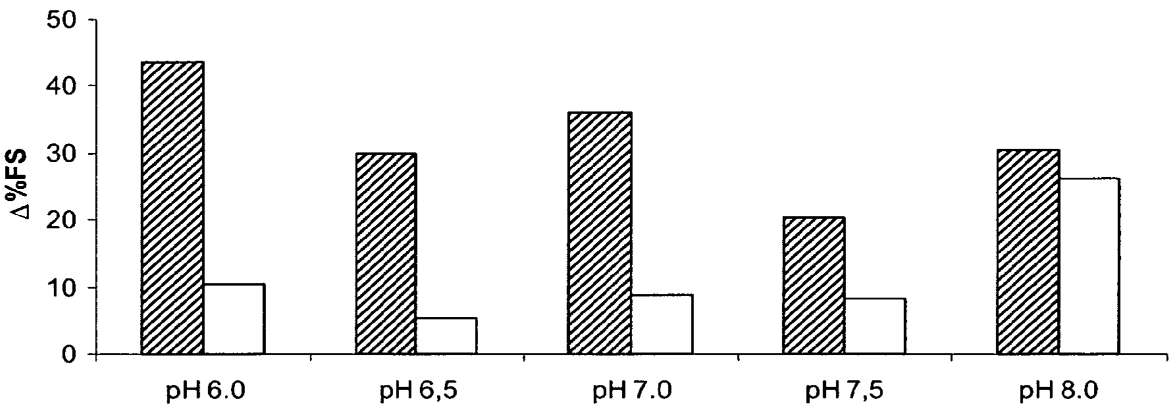


Figure 16

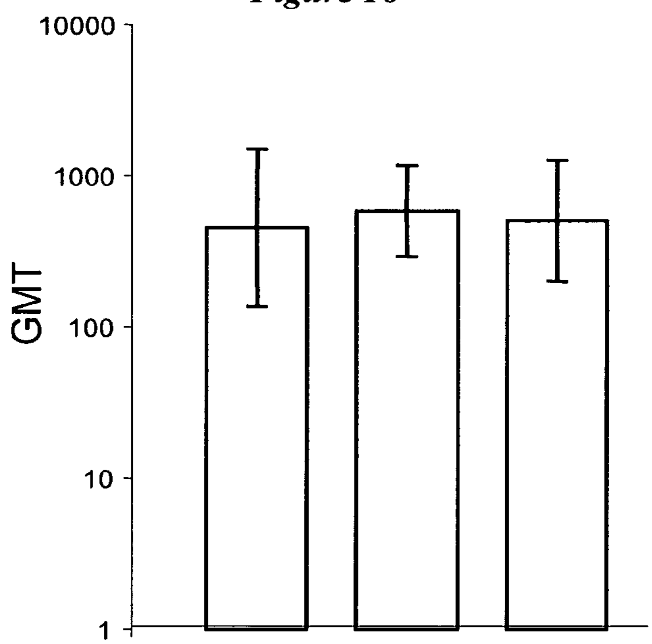


Figure 17

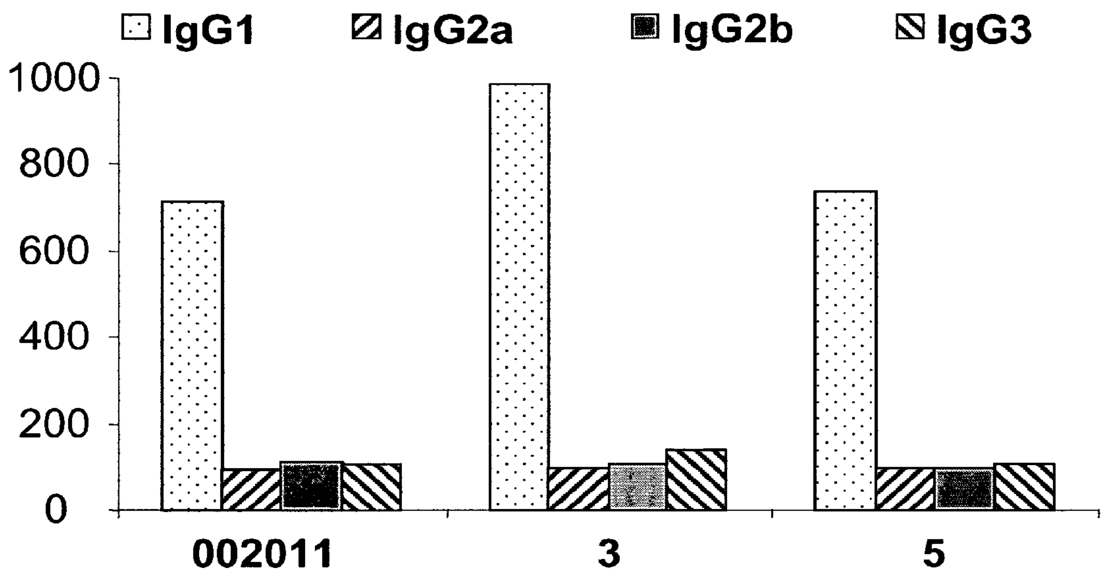
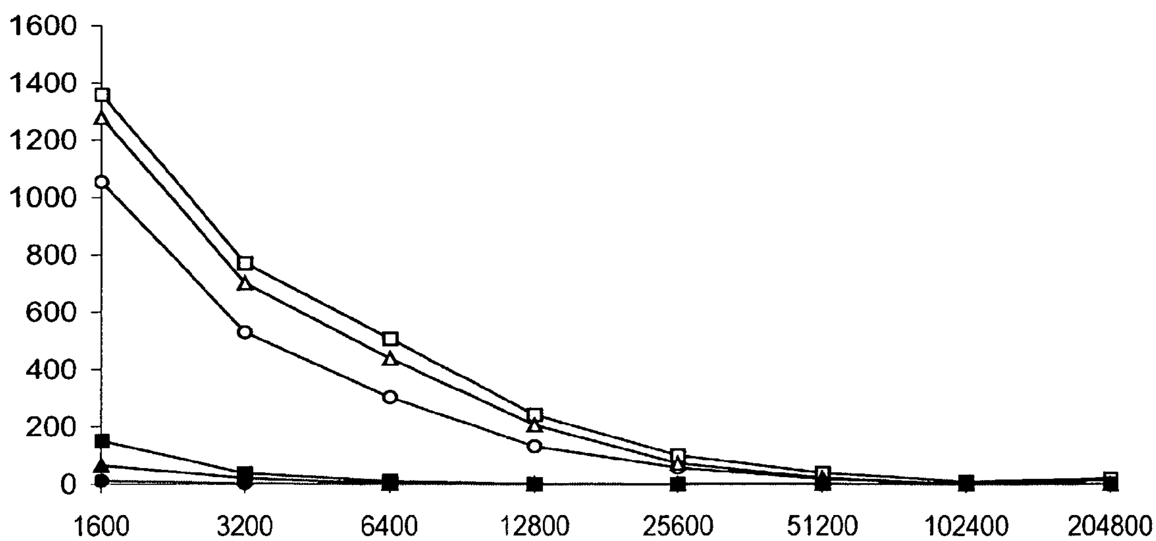


Figure 18



INTERNATIONAL SEARCH REPORT	Inter l Application No PCT/IB 03/01436

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C08B37/00   A61K39/385   A61K39/095

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C08B   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CHEM ABS Data, EMBASE, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 733 459 A (A. WANDER AG) 13 July 1955 (1955-07-13) page 1, line 10 - line 16 page 1, line 90 -page 2, line 17 page 3, line 85 - line 99 page 4, line 25 - line 51 page 5, line 118 - line 129 ---	1,2,5,6, 10-12, 16-21, 34,35
X	WO 98 42718 A (BRIGHAM & WOMEN'S HOSPITAL) 1 October 1998 (1998-10-01) page 9, line 1 -page 10, line 29 claims page 13, line 16 - line 23 --- -/--	1-4,6, 10,15, 20,21, 34,35,50

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 June 2003	03/07/2003
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Mazet, J-F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	GB 2 009 198 A (BEHRINGWERKE AKTIENGESELLSCHAFT) 13 June 1979 (1979-06-13)	
X	WO 93 07178 A (PASTEUR MERIEUX SERUMS ET VACCINS) 15 April 1993 (1993-04-15) page 3, line 15 -page 4, line 7 page 10, line 1 - line 26 page 11, line 14 -page 12, line 17 page 13, line 10 - line 21 page 16 -page 17; example 3 figure 1G	1-6, 10-13, 15-19, 36-51
P,X	WO 03 007985 A (CHIRON SPA) 30 January 2003 (2003-01-30) page 3, line 28 - line 29 claims	1-51
A	US 4 882 317 A (MARBURG ET AL.) 21 November 1989 (1989-11-21) cited in the application column 5, line 44 -column 6, line 2 column 7, line 1 -column 8, line 62	1-51
A	WO 99 32653 A (NORTH AMERICAN VACCINE INC.) 1 July 1999 (1999-07-01) the whole document	1-6,10, 15-21, 34-51
A	WO 98 08543 A (CHIRON CORPORATION) 5 March 1998 (1998-03-05)	
A	US 4 727 136 A (JENNINGS ET AL.) 23 February 1988 (1988-02-23)	

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Inter_nal Application No
PCT/IB 03/01436

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